

Identification of potential marker proteins of toxicant-induced damage to spermatogenesis

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**I would like to dedicate this thesis to the
memory of my father, Bill McLaren**

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Abstract

Spermatogenesis involves a complex series of cell-cell interactions which are probably mediated by secreted proteins. The primary objective of the studies described in this thesis was therefore to identify specific proteins which change in relative abundance in the early stages of toxicant-induced damage to spermatogenesis and which might have potential use as markers of such damage. Identification of such proteins might pin-point the possible biochemical causes of the toxic effects on the testis, and also give insight into normal control mechanisms in spermatogenesis. The chemicals used in these studies were meta-dinitrobenzene (m-DNB), nitrobenzene (NB) and methoxyacetic acid (MAA).

The effect of age on seminiferous tubule (ST) protein secretion and the susceptibility to the adverse effects of testicular toxicants in the rat was assessed first. Addition of m-DNB, NB, MAA or FSH to isolated ST *in-vitro* either had no effect (NB) or had a small stimulatory effect (FSH, m-DNB and MAA) on the overall incorporation of ^{35}S -methionine into proteins secreted by ST isolated from rats aged 28 days. In contrast, the same additions to ST isolated from adult rats resulted in a decrease in protein secretion in every instance. Analysis of ST-secreted proteins by 2-D SDS PAGE revealed a large number of major age-dependent differences in the proteins secreted by ST from immature and adult rats. Most of these proteins were prominent secretory products of ST from adult rats but were minor or non-detectable secretory products of cultures of ST from immature rats. Most of these proteins disappeared or decreased in abundance after culture with m-DNB or NB. Two proteins showed the reverse pattern, being more prominent secretory products in immature than mature rats; secretion of these proteins was unaffected or was increased by toxicant exposure.

As it is known that Sertoli cell secretory function changes according to the stage of the spermatogenic cycle, it is presumed that some of these functions are either altered selectively or are more sensitive to the adverse effects of particular chemicals. Therefore, the early effect of m-DNB, NB or MAA on the secretion of proteins by ST isolated from adult rats at different stages of the spermatogenic cycle was assessed. Within 24 hours of a single oral administration of m-DNB, NB or MAA to adult rats, stage-specific changes in the overall incorporation of ^{35}S -methionine into secreted proteins were observed. Compared to controls, ST at stages VI-VII and IX-XII showed a significant decrease in incorporation, whereas, ST at

showed no change (NB and MAA) or a slight increase in incorporation (m-DNB) of radiolabel into secreted proteins. A similar profile of incorporation was observed after 72 hours pretreatment *in-vivo* with m-DNB, NB or MAA. A similar picture was obtained with ST that had been isolated from control rats and cultured in the presence of m-DNB, NB or MAA (10^{-4} M) for 24 or 72 hours. Analysis of ST-secreted proteins by 2-D SDS PAGE identified ten potential 'markers' of toxicant-induced damage to spermatogenesis following exposure to either m-DNB or NB. Of these ten proteins, seven were found to change following exposure to MAA.

The effect of severe disruption of spermatogenesis, induced by short-term local testicular heating, was assessed in order to establish whether protein changes comparable to those seen following toxicant exposure could be identified. Within 4 hours of treatment, stage-specific changes in the incorporation of 35 S-methionine into both secreted and intracellular proteins were observed. Analysis by 2-D SDS PAGE identified seven proteins which were affected adversely following heat treatment, all of which had been affected by toxicant exposure.

In conclusion, the studies presented in this thesis have identified proteins which have potential use as markers of early toxicant-induced damage to spermatogenesis. Studies to date in the rat have identified proteins in peripheral blood which derive from the Sertoli cells and germ cells and the expectation is that most if not all ST-secreted proteins will appear in blood. Therefore the logical next step will be to determine whether any of the proteins identified in the present studies are detectable in peripheral blood and whether the amounts change following toxicant exposure.

[~60 000 words in main text]

1. Introduction

Infertility affects 5% or more of the male population in Europe and, in the vast majority of cases, the cause is unknown. A recent study by Carlsen *et al.* (1992) has shown that mean sperm counts have fallen by 40–50% over the last half-century. Such a dramatic fall over a relatively short period of time may be attributable to environmental factors (Sharpe & Skakkebaek, 1993), although the extent to which environmental exposure to chemicals has contributed to this overall decrease remains largely unknown because of the practical and ethical problems involved in making such an assessment, both in the general population and in specified 'at risk' workforces (Sharpe, 1992). At present, we lack sensitive endpoints with which to identify early toxic damage to man. Endpoints that are currently used in assessing toxic damage to the testis rely almost exclusively on monitoring changes in testicular weight, blood levels of reproductive hormones (namely, luteinizing hormone, testosterone and follicle-stimulating hormone) and, in some instances, semen profiles. However, all of these parameters are extremely insensitive, in that they can only detect severe damage to spermatogenesis, and then only many weeks after initial exposure to the chemical. This highlights the need for more sensitive methods which would enable us to detect early adverse changes within the testis. Ideally, what is required is a marker which will detect and inform on early toxicant-induced abnormalities in spermatogenesis, detectable preferably in peripheral blood.

It is generally believed that the Sertoli cell, which is intimately associated with the developing germ cells, plays a central role in the process of spermatogenesis via the secretion of several hundreds of proteins which drive the developing germ cells through the many stages of differentiation and maturation. It is also known that the function of Sertoli cells changes in accordance with the stage of development (and thus of the requirements) of the germ cells (Parvinen, 1993). It is presumed that these intimate interactions, between the Sertoli and germ cells, are paracrine in nature. There is substantial evidence, however, which suggests that many of the proteins involved, especially those derived from the Sertoli cells, either 'leak' or are secreted into fluids that leave the testis. It is well recognized that Sertoli cell proteins are secreted in a bidirectional manner i.e. via the base of the Sertoli cell into testicular interstitial fluid (IF) and thence into the bloodstream, or via the apex of the Sertoli cell into seminiferous tubule fluid (STF), and the expectation is that most (and probably all) of the secreted

proteins will appear in semen or blood (see Sharpe, 1988, 1992) and are thus potential candidates for the monitoring of spermatogenesis.

Our understanding is that toxicants are relatively selective in their effects, affecting only particular germ cells and particular stages of the spermatogenic cycle (e.g. Creasy and Foster, 1984; Blackburn *et al.*, 1988; Hess *et al.*, 1988; Allenby, 1990). The primary objective of this thesis was therefore to identify specific proteins, which change in relative abundance, following toxicant exposure. Identification of such proteins would not only highlight the possible biochemical cause(s) of the toxic effects of certain chemicals on the testis, but also give insight into normal control mechanisms in spermatogenesis.

To date, in order to study cell-cell interactions within the testis, most studies have used isolated cells from immature animals and, as the function of the Sertoli cell is known to change with increasing age (Castellón *et al.*, 1989; Jégou, 1991) it is not certain that the results obtained are relevant to the adult animal (Russell and Steinberger, 1989). Therefore, the effect of age on the adverse effects of testicular toxicants was assessed (Chapter 4). Subsequent chapters, using adult animals, have assessed the stage-specific effects of disruption to spermatogenesis either by testicular toxicants, for which the cellular site of action is known (Chapters 5, 6 and 7), or by local testicular heating (Chapter 8). The adverse effects of testicular toxicants on protein secretion by seminiferous tubules isolated from rats and man were also examined in order to establish whether specific proteins comparable to those seen in the rat can be identified in man (Chapter 9).

Each chapter has a specific introductory section relevant to the questions addressed by the experiments described in that chapter, and aims to review the work of others in that particular field. Thus, the overview provided in Chapter 2 does not set out to review the whole literature pertinent to all of the material in this thesis; it aims solely to provide a foundation for the understanding and appreciation of the studies reported. Chapter 2 therefore contains background information on the structure of the testis, its physiology and the complex role played by endocrine hormones and locally produced paracrine factors in spermatogenesis. Particular emphasis is placed on the Sertoli cell and developing germ cells and their interactions within the testis, since the two toxicants used in most of the experiments reported in this thesis are both known Sertoli cell toxicants.

2. Literature Review

2.1. Historical Aspects

Although the effects of castration were recognized many centuries ago, probably as early as Neolithic times (c. 7000 BC), the association between the testis and fertility was not revealed until the seventeenth century. Reasonably accurate diagrammatic descriptions of testicular anatomy can be attributed to Aristotle in 400 BC. In spite of his generally accurate observations, Aristotle maintained that the testes were not necessary for fertility. A treatise by de Graaf in 1608 recorded accurately the general structure and functions of the testis. Shortly thereafter, in 1667, van Leeuwenhoek described the presence of spermatozoa ("animalcules") in seminal fluid. The link between the seminiferous tubules and the production of spermatozoa was not realised fully until the studies of van K  lliker in 1840, who concluded that spermatozoa were formed by a process of cellular development within the seminiferous tubules. The cellular changes resulting in the production of spermatozoa thus constituted spermatogenesis (Leblond and Clermont, 1952).

Accurate descriptions of the hormonal effects of castration were also available in Aristotle's time, but the first experimental evidence emerged from the studies of Berthold (1849), who showed that the decrease in comb size and absence of crowing evident in roosters after castration could be reversed by transplantation of the testis. Berthold concluded that the testis was able to affect the bloodstream which, in turn, could influence other tissues. Although the existence of Leydig cells was known in 1850, it was not until 1903, on the basis of purely morphological evidence, that the Leydig cell was postulated as the site of synthesis of many of the factors responsible for the development of male characteristics (Bouin and Ancel, 1903), but the isolation of testosterone, by David *et al.*, did not occur until 1935.

The non-germinal component of the seminiferous epithelium was described originally by Enrico Sertoli in 1856. He identified the cells that now bear his name as being tall columnar cells extending from the basement membrane lining the tubules to the lumen of the seminiferous epithelium, and enveloping the many clusters of associated germ cells.

Improvements in microscopy in the later nineteenth century expanded the knowledge of the light microscopic features of spermatogenesis, and identification of chromosomes, and the processes of mitosis and meiosis

improved greatly the understanding of gamete production in the male. The results of these studies laid the foundation of our current knowledge.

2.2. Endocrine control of the testis

The initiation, development and maintenance of the mammalian testis are regulated by both endocrine and paracrine control systems. Endocrine control is exercised via the secretion of the pituitary gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) as well as by prolactin, although the role of prolactin is less clear.

2.2.1. FSH

FSH belongs to a family of glycoproteins the structure of which consists of two non-covalently linked subunits, α and β . The 92 amino acid α -subunit is common to other members of this family, namely, LH, thyroid-stimulating hormone (TSH) and human chorionic gonadotrophin (hCG), but each has a unique β -subunit which confers specific biological activity via target tissue receptor recognition. FSH has a greater carbohydrate composition than LH and this accounts for its higher molecular weight and longer half-life (Coble *et al.*, 1969). FSH, like LH, has been shown to be secreted in a pulsatile manner (Negro-Vilar *et al.*, 1986), although the levels of FSH secretion show smaller fluctuations than those of LH (Santen and Bardin, 1973).

2.2.2. Control of FSH secretion by the hypothalamus

The release of FSH and LH from pituitary gonadotrophs is controlled by the pulsatile secretion of gonadotrophin-releasing hormone (GnRH) from neurons in the arcuate nucleus of the hypothalamus (Schally *et al.*, 1971; Knobil, 1980). GnRH has been shown to bind to specific high affinity receptors on gonadotroph cell membranes (Theolyre *et al.*, 1976).

GnRH receptors do not use the classic adenylate cyclase/cAMP second messenger system (Conn *et al.*, 1979). It has been shown that, following receptor binding, GnRH stimulates the activation of phospholipase C (PLC) which is thought to be mediated by a pertussis toxin-insensitive GTP-binding protein. The subsequent rapid formation of inositol - 1, 4, 5 -triphosphate (IP₃) and 1, 2-diacylglycerol (DAG) then enhances calcium release from intracellular stores, probably via a specific IP₃ receptor, and this may be the cause of the translocation of protein kinase C (PKC) subspecies from the

cytosol to the membrane. The activated PKC subspecies in turn phosphorylates substrate proteins, which activate secretory reactions and participate in gonadotrophin gene expression. Parallel Ca^{2+} influxes via nifedipine-sensitive and insensitive channels further elevate intracellular calcium levels, which participate in the sustained phase of gonadotrophin secretion in concert with activated PKCs.

Like many other hormonally controlled tissues, arachidonic acid (AA) and its metabolites play a role in the regulation of pituitary gonadotrophs. GnRH has been shown to trigger the release of AA from gonadotroph phospholipids. Moreover, inhibition of AA release, by blocking phospholipase A_2 activity, prevents GnRH-stimulated LH secretion (Naor and Catt, 1981). The lipoxygenase pathway has been reported to modulate, at least in part, the action of GnRH on pituitary gonadotrophs (Snyder *et al.*, 1983) and it seems that arachidonic acid and its lipoxygenase and/or epoxygenase-derived metabolites might play a role in the cascade of events initiated by GnRH to promote FSH and LH secretion (reviewed by Naor, 1991).

GnRH has been shown to stimulate the release of FSH and LH from enriched populations of gonadotrophs (Benoist *et al.*, 1981). Yen *et al.* (1982) showed that, when synthetic GnRH was administered to normal adult men, an increase in the level of LH was observed, accompanied by a smaller but concomitant rise in circulating FSH levels. A separate study showed, however, that the levels of FSH and LH, following synthetic GnRH administration, were asynchronous (Mortimer *et al.*, 1974). On the basis of these results it may be suggested that divergent pituitary synthesis and release mechanisms for FSH and LH must exist.

2.2.3. Site of FSH action

The first evidence that the Sertoli cell was the primary target site of FSH action was provided by Murphy (1965a,b) who showed that injections of FSH into immature hypophysectomized rats resulted in a change in the secretory activity of the Sertoli cell. Subsequently, Mancini and his colleagues (1967) demonstrated that, following the injection of an electron-dense substrate such as ferritin conjugated to FSH, ferritin was found to localize predominantly over Sertoli cell cytoplasm. To further localize within the seminiferous tubules the site of FSH action, studies were performed using animals in which the testes had been irradiated to form Sertoli cell-only tubules and, using this

technique, it has been shown that the level of binding of FSH to the tubules is almost identical to that seen in normal animals, proving that the Sertoli cell is the site of action of FSH (Means and Huckins, 1974).

FSH has been shown to bind to a cell surface receptor on Sertoli cells. Recently, the FSH receptor has been cloned by screening a library of Sertoli cell cDNA with oligonucleotide probes that were obtained from portions of the LH receptor. The sequence of 675 amino acids, derived from the cDNA sequence, showed that the receptor is a member of the 7-transmembrane helix-G protein coupled receptor family (Sprengel *et al.*, 1990). The human FSH receptor cDNA has been isolated also and shows a 89% overall homology to the sequence of the receptor from the rat (Minegis *et al.*, 1990). Like many other protein hormones FSH, when bound to its receptor, induces the internalization and degradation of the hormone-receptor complex.

2.2.4. Mechanism of FSH action

The interaction of FSH with its receptor, on the membrane of Sertoli cells, results in an increase in cAMP production (Kuehl *et al.*, 1970), which then rapidly activates protein kinase, and subsequent protein phosphorylation. FSH has been shown to exert a number of generalized effects on the Sertoli cells, such as to increase their size, DNA and protein synthesis, and seminiferous tubule fluid production; *in-vitro*, FSH can alter the shape and morphology of Sertoli cells (Means *et al.*, 1976; Rich and de Kretser, 1983; Griswold, 1993). The response of the Sertoli cell to FSH is age-dependent and varies with the developmental status of the animal.

FSH produced by the fetal and early postnatal pituitary is of critical importance in stimulating the proliferation of Sertoli cells. It has been shown that the mitotic index of Sertoli cells is maximal in 19-21 day old rat fetuses, declining steadily thereafter until the 2nd week after birth when cell division ceases (Orth, 1982, 1984; Almirón and Chemes, 1988). FSH is the most important regulator of Sertoli cell replication during fetal and neonatal life and the reduction in Sertoli cell numbers which occurs as a result of the suppression of FSH (Huhtaniemi *et al.*, 1986) and the reversal of this effect following the administration of FSH (Orth, 1982, 1984; Almirón and Chemes, 1988) support this conclusion. Furthermore, hemicastration of immature rats results in an increased proliferation of Sertoli cells in the contralateral testis, coincident with increased serum levels of FSH (Orth *et al.*, 1984).

Most of the studies which have examined the actions of FSH have been performed using Sertoli cells which have been isolated from rats aged between 10 and 30 days. In these studies, FSH has been shown to increase levels of cAMP and protein synthesis, in addition to stimulating Sertoli cell products such as androgen-binding protein (Fritz *et al.*, 1976; Louis and Fritz, 1979; Hall *et al.*, 1990), inhibin (Morris *et al.*, 1988; Risbridger *et al.*, 1989; Toebosch *et al.*, 1989; Pineau *et al.*, 1990), plasminogen activator (Lacroix *et al.*, 1977; Hettle *et al.*, 1986) and lactate (Mita *et al.*, 1982; Jutte *et al.*, 1983).

With increasing age, maturation of the Sertoli cells brings about an age-dependent decline in responsiveness of the Sertoli cell to FSH in terms of the secretion of cAMP, ABP, transferrin, inhibin and STF production (Means *et al.*, 1976; Rich *et al.*, 1983; Jégou *et al.*, 1982, 1983; Le Magueresse *et al.*, 1986; Le Magueresse and Jégou, 1988a; Sanborn *et al.*, 1986; Castellón *et al.*, 1989a). Although levels of FSH in blood fall quite markedly during puberty in the rat (Ketelslegers *et al.*, 1978; Sharpe *et al.*, 1988; Maddocks and Sharpe, 1990), the decreasing responsiveness of the Sertoli cell to FSH is thought not to be related directly to this or to a reduction in the number of FSH receptors, as it has been shown that receptor numbers actually increase as a function of testicular maturation (Ketelslegers *et al.*, 1978); rather it appears to be due to the large increase in the cAMP-phosphodiesterase activity within the cell (Ritzén *et al.*, 1989), as responses to FSH in Sertoli cells isolated from adult rats can be measured usually only in the presence of a phosphodiesterase inhibitor.

Although there is general agreement that FSH is of critical importance in the initiation and expansion of spermatogenesis in mammals during puberty (Means *et al.*, 1976; Dym *et al.*, 1979; Russell *et al.*, 1987), its role in the adult male is the subject of much debate. Much of this debate has resulted from the apparently different results obtained in rodents and primates following immunoneutralization of FSH. Active or passive immunoneutralization of endogenous FSH in adult rats using antibodies to FSH or its β -subunit has relatively little or no effect on spermatogenesis (Davis *et al.*, 1979; Dym *et al.*, 1979) whereas, in adult rhesus monkeys, active or passive immunization against FSH leads to a major suppression of spermatogenesis and a considerable decrease in sperm output within four weeks of immunization (Wickings and Nieschlag, 1980). Furthermore, Matsumoto *et al.* (1986) have demonstrated that, in man, FSH is necessary for the maintenance of quantitatively normal spermatogenesis. In this study normal men were treated twice weekly for 4 to 7 months with hCG. This treatment

maintained normal or raised levels of testosterone but suppressed FSH levels by more than 90%. The sperm count of the men treated was shown to decrease by over 80% during hCG treatment but could be returned to within control levels by the concomitant administration of FSH. Using a highly purified preparation of human FSH, Bartlett *et al.* (1989) demonstrated that treatment of hypophysectomized rats with this preparation maintained numbers of preleptotene and pachytene spermatocytes and round spermatids, at levels higher than those seen in untreated hypophysectomized rats. Testosterone implants were also able to maintain spermatogenesis, but, FSH in combination with testosterone was able to maintain spermatogenesis far more effectively than either hormone alone.

A model for the synergistic action of FSH and testosterone in maintaining spermatogenesis was proposed by Sharpe (1989a); FSH is thought to determine the number of cells available to enter meiosis, while testosterone controls specific stages of their subsequent development. It follows, therefore, that FSH on its own would be unable to maintain spermatogenesis (see Bartlett *et al.*, 1989), as continued development of later germ cells would fail due to the lack of androgen support. The complex co-operative effects of FSH and testosterone upon the seminiferous epithelium are emphasized by the results of a study by Kerr *et al.* (1992) who showed that the effect of FSH was manifest at stages I-VI of the spermatogenic cycle, whereas progression of the meiotic and post-meiotic germ cells beyond stage VI was dependent upon the presence of testosterone. The stage-specificity of FSH action on spermatogenesis is consistent with the stage-dependent expression of FSH receptors and FSH responsiveness as measured by the levels of adenylate cyclase and cAMP production (Parvinen *et al.*, 1980; Gordeladze *et al.*, 1982; Kangasniemi *et al.*, 1990a,b).

Another way in which FSH might support spermatogenesis, in the adult, is in a secondary manner via the Leydig cells. There is evidence from both rodents and man that FSH can stimulate the production of paracrine factors, by the Sertoli cells, which can act on the Leydig cells, thereby enhancing testosterone production in response to LH (Verhoeven and Cailleau, 1987). FSH is also implicated in the regulation of Leydig cell development. For example, in the immature rat testis, the absence of exposure to FSH results in insensitivity, which suggests that exposure to FSH induces responsiveness to LH (Odell *et al.*, 1973). Furthermore, Kerr and Sharpe (1985) reported that administration of FSH to hypophysectomized immature rats increases the size and number of Leydig cells and appears to

induce the differentiation of Leydig cells which are morphologically and functionally (Murone and Payne, 1979) adult in type.

2.2.5. Feedback control of FSH secretion

Several studies have indicated that both testosterone and inhibin are important in the control of FSH secretion *in-vivo*, as it has been shown that administration of testosterone to castrated rats results in a decline in the secretion of FSH and LH (Decker *et al.*, 1981). Furthermore, when testosterone esters are administered by injection in low or high doses to intact rats, serum FSH is reduced to low or undetectable levels in these rats (Sharpe *et al.*, 1988a,b). Studies in man involving comparable treatment with low doses of testosterone esters have reported identical findings (Matsumoto *et al.*, 1986). Further evidence of the dual control of FSH secretion has arisen from experiments which have utilized the Leydig cell cytotoxin, ethane dimethane sulphonate (EDS). In these experiments, falling testosterone levels are accompanied by increasing FSH and LH levels, suggesting a role for testosterone in the control of FSH secretion (Jackson *et al.*, 1986). It is important to note, however, that FSH levels were elevated to only 50% of the levels detected in castrated animals, suggesting the existence of an intratesticular factor which inhibits further rises in FSH. Furthermore, in bilaterally cryptorchid rats, the rise in serum FSH levels to approximately 50% of the levels seen in castrate animals has been attributed to diminished inhibin secretion as serum testosterone remains at normal levels (Au *et al.*, 1983). The concept of dual control of FSH secretion by inhibin and testosterone was supported further by studies in which EDS was given to cryptorchid animals. The action of EDS in cryptorchid animals results in the suppression of testosterone (due to Leydig cell destruction) and a rise in FSH levels to within the range observed in castrated animals. Following the redevelopment of new Leydig cells 14-21 days later, testosterone levels were restored and FSH levels fell to their pre-treatment (cryptorchid) level (O'Leary *et al.*, 1986). These data show that, in the rat, under a number of experimental conditions, the control of FSH can be attributed to both testosterone and inhibin.

Previous studies have demonstrated, however, that during maturation in the rat, blood levels of inhibin decline steadily through puberty and into adulthood (Rivier *et al.*, 1988; Culler and Negro-Vilar, 1988; Maddocks and Sharpe, 1990) and it has been suggested that this reflects a reduced and

altered route of secretion of inhibin by the Sertoli cells. In the normal adult rat, the question that must be asked is, what contribution does inhibin make in controlling FSH secretion? Culler and Negro-Vilar (1988) demonstrated that the administration of an anti-serum directed against the inhibin α -subunit to neonatal male rats caused a dramatic increase in plasma FSH concentrations. However, when the same antiserum was administered to adult male rats, no rise in FSH occurred. These results suggest that inhibin plays an important role in suppressing FSH secretion in neonatal rats, whilst testosterone is the over-riding factor in controlling FSH output in the pubertal and adult rat. More recently, Culler and Negro-Vilar (1990) showed that inhibin immunoneutralization and selective removal of Leydig cells, in the adult rat, with EDS, resulted in a significant rise in FSH, indicating a complex relationship between testosterone and inhibin in the control of FSH secretion.

Whilst it has been recognized that Sertoli cells are the source of inhibin in the male, a recent study by Risbridger *et al.* (1989a) has shown that rat Leydig cells produce both immuno- and bioactive inhibin in culture and this may contribute to the levels measured *in-vivo*. Maddocks and Sharpe (1989b) evaluated this contribution, in rats in which the Leydig cells had been destroyed by an injection of ethane dimethane sulphonate (EDS). In these animals, it was found that inhibin levels increased in testicular interstitial fluid (IF), and in testicular (TV) and spermatic venous (SV) blood. In EDS-treated rats which had been supplemented for 21 days with testosterone esters, significant changes occurred in the levels of inhibin in IF, in TV and SV plasma, and in the route of secretion of inhibin from the testis. However, none of these changes was related to the presence or absence of Leydig cells. Maddocks and Sharpe (1989b) concluded that Leydig cells make little contribution to the intratesticular and blood levels of inhibin in the rat.

2.2.6. LH

LH belongs to the same glycoprotein family as FSH and is also secreted by pituitary gonadotrophs. LH and FSH share a common α -subunit, but the β -subunit of LH is unique. The approximate molecular weight of LH is 28kDa, of which 12-14% is carbohydrate. In all mammals studied, including humans, the secretion of LH by the pituitary is pulsatile. In man, LH pulses occur approximately every 90 minutes (Nankin and Troen, 1971; Santen and Bardin, 1973). In the rat the timing of LH pulses can be variable (Ellis and Desjardins, 1982).

2.2.7. Control of LH secretion by the hypothalamus

The episodic nature of LH secretion by the pituitary is controlled by the pulsatile secretion of GnRH from the arcuate nucleus of the hypothalamus. Since the pituitary has no intrinsic pulsatility, pulsatile secretion of GnRH is responsible for the episodic bursts of LH secretion. This has been demonstrated directly by Carmel *et al.* (1976) who measured episodic GnRH secretion in the hypophyseal-portal blood of female Rhesus monkeys and observed peak intervals of between 1-3 hours. Ellis *et al.* (1983) administered either a GnRH antagonist, or ovine antiserum to GnRH, to castrated male rats and found that both treatments were able to arrest pulsatile LH secretion.

2.2.8. Site of LH action

Luteinizing hormone was first purified in the 1930's and its availability provided the means of demonstrating that LH acted upon the Leydig cells (Greep *et al.*, 1936), as it was shown that this preparation could maintain normal Leydig cell morphology after hypophysectomy. Subsequently, Mancini *et al.* (1967) demonstrated that, when LH was labelled with various histochemical agents, it was localized to Leydig cells of adult rats. During the same period studies demonstrating *in-vivo* localization of iodinated LH and the *in-vitro* binding of LH by rat testes homogenates were also carried out (de Kretser *et al.*, 1969).

LH has been shown to bind to a cell surface receptor on the Leydig cell (Hsueh *et al.*, 1976). Recently the LH receptor has been cloned from human and rat ovaries and porcine Leydig cells. Its sequence indicates that it is a member of the 7-transmembrane helix-G protein coupled receptor family (Loosfelt *et al.*, 1989; McFarland *et al.*, 1989). The receptor has a large extracellular domain which is the site of hormone binding. It is now known that there is a rapid turnover of LH receptors and that, following binding, the receptor-hormone complex is internalized and recycled and/or down-regulated (Habberfield *et al.*, 1987; Cooke and West, 1992).

There is unequivocal evidence showing that LH is able to down-regulate the number of its own receptors (Sharpe, 1984), as it has been shown that injections of LH or hCG into immature or adult control or hypophysectomized rats cause a dose-dependent decrease in the number of available LH receptors (Sharpe, 1982). It is well established, however, that all Leydig cells contain spare LH receptors (Catt *et al.*, 1979) and that less than 1% of the available receptors need to be occupied in order to maximally

stimulate steroidogenesis. Thus, the function of receptor down-regulation might therefore be to reduce sensitivity to LH-stimulation without reducing the capacity of the Leydig cells to respond (Raff, 1976).

2.2.9. Mechanism of LH action

LH acts exclusively on Leydig cells and is the primary regulator of testosterone production. Testosterone has both intratesticular actions and multiple effects on reproductive and non-reproductive organs (Sharpe, 1986). LH therefore represents the vehicle by which the brain is able to exert overall control of the timing of testosterone production.

The binding of LH to specific receptors, located on the plasma membrane of Leydig cells, results in the production of cAMP via the activation of membrane-bound adenylate cyclase (Kuehl *et al.*, 1970; Dorrington and Fritz, 1974). The intracellular production of cAMP initiates protein phosphorylation through the activation of protein kinases. Dufau *et al.* (1977) demonstrated that the increasing testosterone response of purified rat Leydig cells to increasing concentrations of hCG was accompanied by a simultaneous increase in the levels of cAMP. Similarly, increasing concentrations of cAMP can be found in the spermatic vein within 5 minutes following hCG administration in humans (Yasukawa *et al.*, 1981). The endpoint of the action of LH appears to be the regulation of the cytochrome P450 mitochondrial cholesterol side chain cleavage enzyme.

It is not certain that all of the effects of LH are mediated via the cAMP second messenger system, as many investigations have shown that testosterone production can be stimulated by amounts of LH which cause no detectable changes in the levels of cAMP (Cooke *et al.*, 1976). In addition to the stimulatory action of LH on steroidogenesis, prolonged exposure to LH causes Leydig cell desensitization. A feature of this LH-induced desensitization of Leydig cells is that, although these cells become refractory to further LH stimulation, they still have a high continuous production of 'basal' cAMP, which is approximately 50% of the levels obtained with the maximum stimulating levels of LH and which is not due to residual receptor-bound LH. This effect cannot be mimicked by cAMP analogues, but can be mimicked by protein kinase C activators such as phorbol esters (Rose and Band, 1988). Further studies led Rose and Band to propose that the initial activation of adenylate cyclase may be dependent upon the activation of PKC.

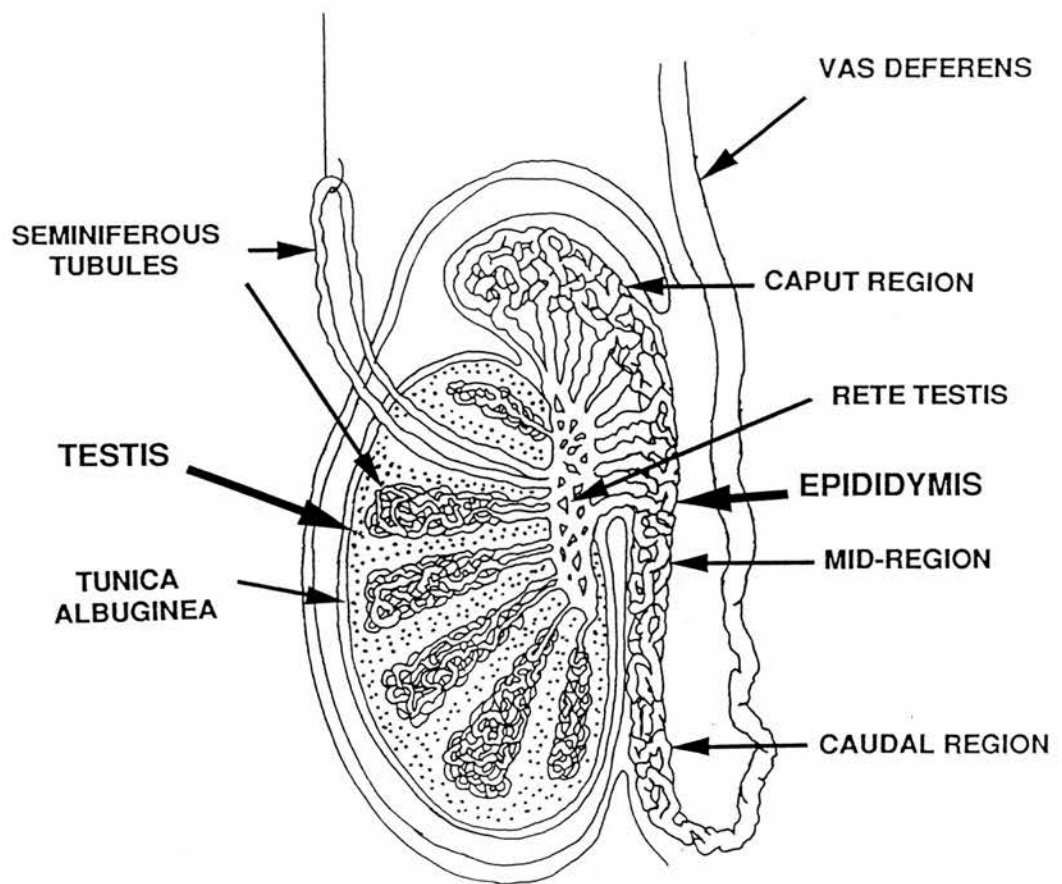
LH has been shown to stimulate the release of arachidonic acid from Leydig cells. Arachidonic acid can be released from phospholipids by calcium-mediated activation either of phospholipase A₂ (PLA₂) or of phospholipase C (PLC), followed by hydrolysis of inositol- 1, 4, 5-triphosphate (IP₃) and 1,2-diacylglycerol (DAG). It is then further metabolized via the cyclooxygenase and lipoxygenase pathways to prostaglandins or leukotrienes respectively. It is thought that the products of the lipoxygenase pathway are involved in the control of steroidogenesis (Sullivan and Cooke, 1985). Furthermore, it has been demonstrated that inhibition of PLA₂ inhibits LH-stimulated steroidogenesis without affecting LH-induced cAMP production (Abayasekara *et al.*, 1990). Based upon these results it would seem that physiological levels of LH activate a transducing system that does not involve the formation of cAMP. Instead, it would appear that LH mobilizes calcium either from intracellular stores and/or by opening plasma membrane ion channels. The increased calcium levels activate PLA₂, thereby stimulating the formation of arachidonic acid metabolites which may act as second messengers. At high levels of LH, however, cAMP is stimulated and may then play a co-operative role in steroidogenesis by enhancing the calcium-regulated pathways, particularly via PLA₂ (Cooke, 1990).

2.3 General structure of the testis

The parenchymal tissue of the testis is enclosed within a dense connective-tissue capsule, the tunica albuginea and can be divided into two functional compartments; the avascular seminiferous tubules which comprise over 80% of the testicular mass in most mammals, and the vascularized interstitium, which comprises about 16% of the testicular mass and contains the Leydig cells, macrophages and other minor cell components.

Spermatogenesis takes place in the seminiferous tubules, which are long convoluted cylindrical structures containing two fundamentally different cell populations; the mesoderm-derived Sertoli cells and the various generations of proliferating and differentiating germ cells. The seminiferous tubules are connected at either end to the rete testis, which transports spermatozoa released from the seminiferous epithelium to the epididymis via efferent ducts for subsequent maturation and storage (Figure 2.1). Surrounding the tubules is a layer of peritubular myoid cells, which is, in

Figure 2.1. Diagrammatic representation of the structure of the adult human testis and epididymis. (Reprinted from Allenby, 1990).



turn surrounded by the interstitium. The interstitium contains Leydig cells fibroblasts, vascular and lymphatic vessels and interstitial fluid (IF) (a filtrate of blood plasma-Setchell and Sharpe, 1981) which bathes the seminiferous tubule) (for reviews see, Christensen, 1975; Clark, 1976; Connell and Connell, 1977; Ewing and Zirken, 1983). The major function of the Leydig cell is the production of testosterone required to support the initiation and maintenance of spermatogenesis.

2.3.1. The seminiferous epithelium

The seminiferous epithelium is a complex stratified epithelium containing tall columnar Sertoli cells and various populations of germ cells undergoing proliferation and differentiation. Germ cells are either completely or partially surrounded by the Sertoli cells which rest upon the basal lamina and extend apically towards the lumen of the seminiferous tubule. The close morphological association between Sertoli cells and germ cells suggests that the Sertoli cells have an important role to play in spermatogenesis.

2.3.1.1. The Sertoli cell

The Sertoli cells are the only non-germinal elements within the seminiferous tubule. They were first described by Enrico Sertoli in 1865 as tall, columnar cells with cytoplasmic processes extending from the basement membrane to the lumen of the seminiferous tubule, which envelops the closely associated germ cells; this provides an arrangement whereby each germ cell is contacted or supported by a number of adjacent Sertoli cells (Fawcett, 1975). Sertoli postulated that "the function of these branched cells is closely linked to the formation of spermatozoa".

Sertoli cells of mammals have a different morphological appearance in fetuses and newborns than in adults (Clermont and Perey, 1957). Also called 'supporting cells' these comprise, at birth, practically the entire periphery of the sex cords, the gonocytes being centrally placed. In the rat, at about 35 days of age Sertoli cells display most of the ultrastructural characteristics typical of adult Sertoli cells (Chemes *et al.*, 1979). These characteristics have been described in a number of different species (for reviews see Fawcett, 1975, de Krester and Kerr, 1988). In all species studied, adult Sertoli cells contain a large and irregular nucleus usually situated in the basal portion of the cell. These nuclei are characterised by a homeogenous nucleoplasm which contains primarily euchromatin and a distinctive tripartite nucleolus. The

Sertoli cell contains an abundance of cytoplasm which shows a polarized distribution; the basal and lower trunk regions of the cytoplasm contain an abundance of organelles and inclusions, whereas the apical extensions usually exhibit a paucity of such structures. Exceptions to this rule can be illustrated by preferential distributions of mitochondria, smooth endoplasmic reticulum and glycogen to the uppermost apical extensions of the Sertoli cell cytoplasm in the mouse, squirrel and human (Schulze, 1974; Ross, 1976; Vogl, 1983). Sertoli cell cytoplasm also contains variable amounts of dense bodies, usually referred to as collections of lysosomes, multivesicular bodies and heterophagic vacuoles (Fawcett, 1975), rough endoplasmic reticulum, Golgi apparatus and numerous spindle-shaped mitochondria. As would be expected of a cell that is required to alter its shape radically, all Sertoli cells studied thus far have an elaborate cytoskeleton, together with contractile elements which occupy most parts of the cytoplasmic matrix. In general, the large Golgi apparatus and other cellular components indicate that the Sertoli cell is involved actively in the secretion of proteins, but no obvious secretory granules or vesicles have been identified, suggesting that proteins are not stored, but rather are secreted immediately after synthesis (Tindall *et al.*, 1985).

In addition to the morphological evidence of protein secretion, there are a number of other structural features of Sertoli cells which relate to their function in the seminiferous epithelium. These include: a) numerous cytoplasmic processes which envelop and inter-digitate between developing germ cells (except spermatogonia) to provide both physical support and communication between adjacent Sertoli cells throughout the seminiferous epithelium (Russell, 1980), b) the ability to translocate germ cells from the basement membrane, through the epithelium, to the lumen between adjacent Sertoli cells (Russell, 1980), c) the ability to phagocytose residual bodies (i.e. residual germ cell cytoplasm remaining after spermiation), or abnormal germ cells which degenerate during development (Russell and Clermont, 1977), and d) unique functional specializations between Sertoli cells which maintain the structural integrity of the seminiferous epithelium and constitute the epithelial component of the blood-testis barrier.

2.3.1.2. Germ cell differentiation

The process of germ cell differentiation takes place within the seminiferous tubule and consists of three distinct processes; spermatogonial multiplication, meiosis and spermiogenesis.

The cells that divide by mitosis and constitute the pool of cells which then enter meiosis and spermiogenesis are termed spermatogonia. Spermatogonia are diploid cells which derive from the gonocytes of the fetal testis. Morphologically, three types of spermatogonia can be distinguished in the rat (type A, intermediate and type B) and two in man, (type A and type B). During the first phase type A₀ spermatogonia, located in the basal compartment, undergo mitotic division to yield one of two products - new type A₀ spermatogonia or spermatogonia destined for differentiation. The latter undergo six mitotic divisions to yield in succession A₁, A₂, A₃, A₄, intermediate and type B spermatogonia, which then divide further to form preleptotene spermatocytes. The latter lose contact with the basal lamina, and engage in DNA synthesis (Parvinen *et al.*, 1991), and the subsequent condensation of their chromosomes characterizes the leptotene stage, where thin threads of chromatin are visible. Pairing of homologous chromosomes occurs at the zygotene stage, followed by shortening and thickening of the chromosomes at the pachytene stage, where the primary spermatocytes are enlarged considerably. Crossing over of chromatids and exchange of genetic information occurs during this stage. RNA synthesis occurs especially in mid-to-late stage pachytene spermatocytes (i.e. those at stages VI-VII in the rat, Monesi, 1965). A short diplotene step occurs, followed by the first meiotic division, which yields secondary spermatocytes. These cells undergo a further division to form haploid (round) spermatids. Spermiogenesis is a complex series of events whereby each round spermatid differentiates into a spermatozoon. Spermiogenesis can be separated into three phases. The first phase involves the formation of the acrosome on the anterior surface of the head. The acrosome is a store of enzymes which enables the spermatozoon to penetrate the zona pellucida of the oocyte. The second phase involves nuclear condensation, during which, histones within the nuclear chromatin are replaced by transition proteins, which are in turn replaced by protamines (reviewed by Hecht, 1990). The nucleosome type of chromatin organization is converted into smooth compact chromatin fibres (at steps 15-17; Ward and Coffy, 1991). As a result of these changes, the transcriptionally active nucleus is converted into the quiescent nucleus of the spermatozoa. Therefore, all of

the mRNAs which code for proteins which are required during spermiogenesis beyond step 8 must be transcribed earlier. The result is that many unusually long lived mRNAs are made during spermatogenesis, some of which may be made as early as mid-pachytene when RNA synthesis is greatest (Monesi *et al.*, 1978). The third and final phase involves elongation of the spermatid, which is initiated at about the same time as nuclear condensation but continues up to step 14-15. This phase is characterized by the development of a tail, and the arrangement of mitochondria into a helix which forms the mid-piece of the spermatozoon (Rossen-Runge, 1962). The time taken for a differentiated spermatogonium to become a mature spermatozoa is approximately 8 weeks in the rat. In this species a second wave of spermatogonial differentiation is initiated approximately every 13 days, the interval between two such successive waves of development constituting the duration of the spermatogenic cycle. The process of spermatogenesis is depicted in Figure 2.2. As the process of germ cell maturation proceeds, developing germ cells are translocated from the basement membrane of the seminiferous tubule upwards towards the lumen, resulting in the gradual displacement of successive generations of germ cells. After their release from the seminiferous epithelium, fully mature step 19 spermatids (i.e. the spermatozoa) undergo major maturational changes during their transit through the epididymis, including the acquisition of a progressive motility pattern and the development of the capacity to fertilize an oocyte. The maturation process depends on a series of interactions between spermatozoa, secreted proteins and other factors from the epididymal epithelium (Yanagimachi, 1988).

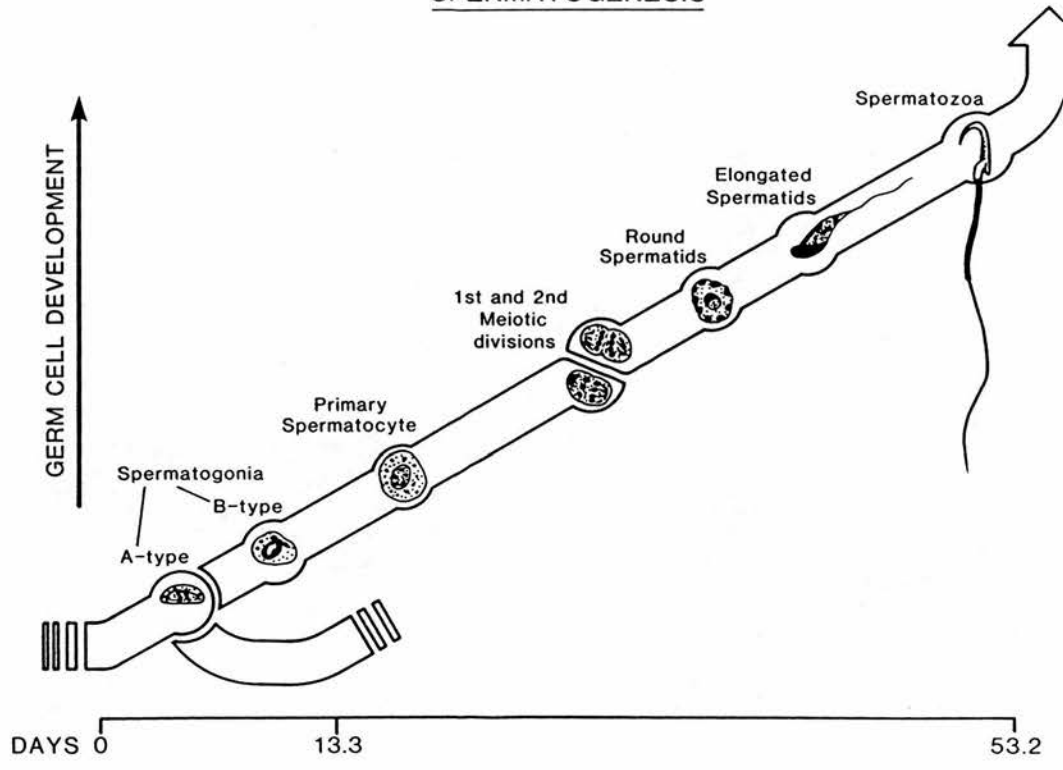
2.3.1.3. Organization of spermatogenesis

In the seminiferous tubule the different generations of developing germ cells are not distributed randomly but are arranged in specific cellular associations or stages. Each stage is composed of four or five generations of germ cells at different stages of their development including one or two generations of spermatogonia, spermatocytes and spermatids which evolve synchronously through the spermatogenic process.

The spermatogenic wave of the rat was separated originally into 8 stages (Roosen-Runge and Giesel, 1950), but the most widely accepted classification now separates the wave into 14 (labelled I-XIV in the rat) distinct stages based on the nuclear morphology of spermatids (Leblond and

Figure 2.2. Diagrammatic representation of spermatogenesis in the rat illustrating the time taken for a differentiated spermatogonium to become a mature spermatozoa. In this species a second wave of spermatogonial differentiation occurs approximately every 13 days. (Reprinted from Lamb and Foster, 1988).

SPERMATOGENESIS



Clermont, 1952), which succeed each other with time in a given area of the seminiferous epithelium. The succession of a complete series of stages constitutes a cycle of the seminiferous epithelium. In the rat it has been shown that each stage of the spermatogenic cycle generally comes into contact only with the stage numerically adjacent to it (thus, stage VIII will always be found between stages VII and IX. The duration of individual stages is constant but varies between stages i.e. stage III lasts 6 hours whilst stage VII lasts 62.8 hours (Figure 2.3).

Based on the classification of the rat seminiferous epithelium described by Leblond and Clermont (1952), the wave of the seminiferous epithelium can be visualized by transillumination in freshly isolated seminiferous tubules (Parvinen and Vanha-Perttula, 1972). When seminiferous tubules are observed under a stereomicroscope, their light absorption is found to vary. An increase in light absorption is associated with the condensation of the chromatin of the spermatid nuclei at step 12 of spermiogenesis. Therefore, stages IX-XII have a pale absorption pattern. Stages XIII-I of the cycle are characterized by a weak absorption. A marked increase in intensity occurs at stage II, concomitantly with a deep penetration of the step 16 spermatids into the Sertoli cells and an increase of their absorption due probably to the development of the outer dense fibres into their flagella. Stages II-V of the cycle are characterized by a strong spot absorption pattern due to deep penetration of steps 16-17 spermatid bundles into the seminiferous epithelium. At stage VI, the bundle arrangement of the step 18 spermatids is released and the cells move centripetally in the seminiferous epithelium to be located at its lumen at stages VII and VIII. This is reflected by a dark homogeneous central absorption. At stage VIII of the cycle, the dark absorption stops abruptly, due to release of spermatids, and the pale absorption pattern reappears (Figure 2.4). Biochemical and endocrinological studies of the seminiferous epithelium became possible when the transilluminated technique was coupled with a microdissection procedure (Parvinen and Ruokenen, 1982). Using this technique a number of hormonal and biochemical differences have been described between the different stages of the spermatogenic cycle (for review see Parvinen, 1982, 1993), suggesting strongly that the Sertoli cells undergo a functional cycle.

Whilst the general organization of the spermatogenic cycle is essentially the same in all mammals, including primates (Chowdhury and Marshall, 1988) in man the human seminiferous epithelium had been regarded as somewhat irregular (Roosen-Runge and Barlow, 1953), as the

Figure 2.3. Diagrammatic representation of the spermatogenic cycle in the rat to illustrate the particular association of the germ cell types present in the seminiferous epithelium, at each stage of the spermatogenic cycle, and the sequential distribution of these stages along a segment of the seminiferous tubule. Each stage lasts for a fixed period of time at the end of which each germ cell type within that stage will have developed into a germ cell characteristic of the following stage. (Adapted from Parvinen, 1982).

The Spermatogenic Cycle

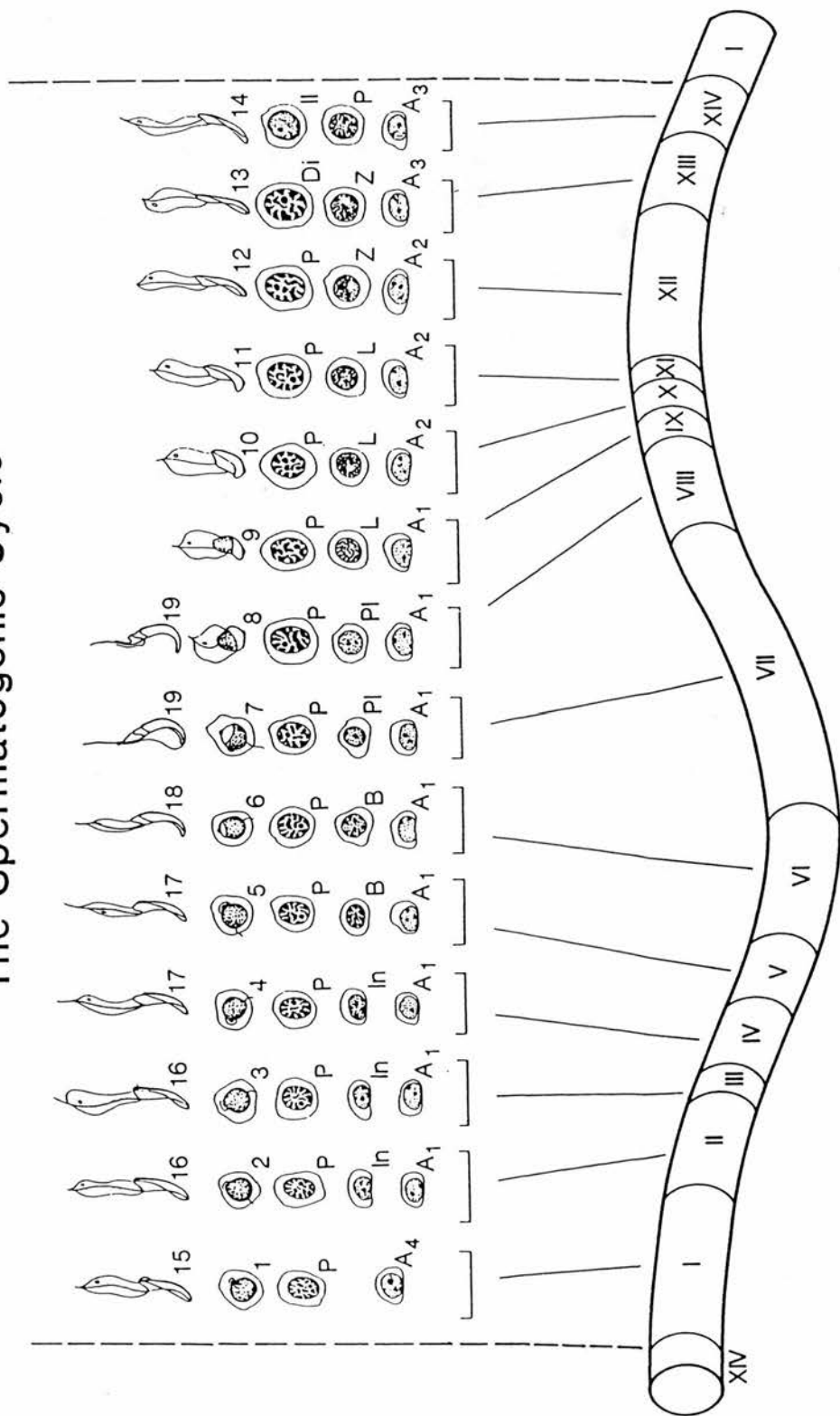
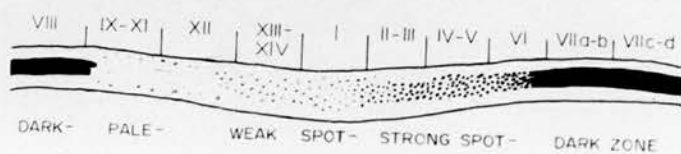
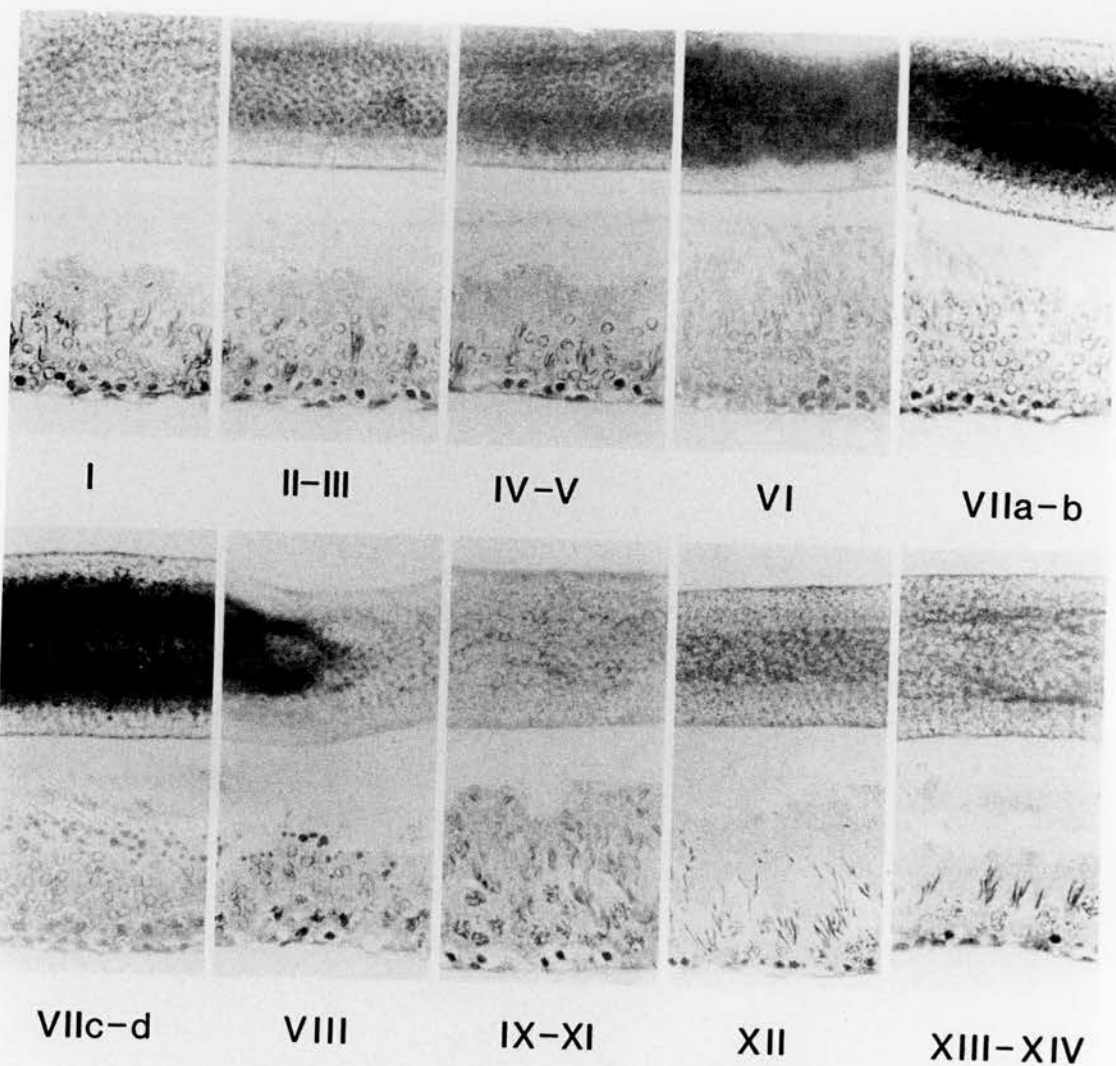


Figure 2.4. Transillumination pattern of the 14 stages of the spermatogenic cycle. When seminiferous tubules are observed under a stereomicroscope, their light absorption is found to vary characteristically from pale absorption to a dark homogenous central absorption. Spermiation is seen as an abrupt stop of the dark absorption at stage VIII. The variation in light absorption is due to differences in the degree of condensation of the nuclei of elongating spermatids and their positions within the seminiferous epithelium (x100). (Reprinted from Parvinen, 1982).



stages appeared to be smaller than in other species and frequently exhibited variability in cell compositions (Clermont, 1963). In addition, a wave-like arrangement of stages was at first not recognized (Heller and Clermont, 1964; Leidel and Waschka, 1970). However, recent cytological, ultrastructural and computer-modelling studies have shown that the arrangement of germ cells within the seminiferous tubule is in fact organized into a helical pattern, based on the geometry of Archimedian circles (Schulze and Rehder, 1984; Schulze *et al.*, 1986), there being six morphologically distinguishable stages of the spermatogenic cycle on the basis of current assessments.

2.3.1.4. Cyclic structural changes in Sertoli cells

The fact that Sertoli cells undergo cyclic structural changes was first suggested in 1901 by Regaud, who reported changes in the shape of the Sertoli cell and nucleus as well as movements of the latter, to and from the basement membrane, during the cycle of the seminiferous epithelium. Regaud reported also that there were cyclic variations in the amount and distribution of secretory vacuoles and lipid droplets. Later, using their classification of cell associations of the seminiferous epithelium in the rat, Leblond and Clermont (1952) reported changes in shape and displacement of the Sertoli cell nucleus at specific stages of the spermatogenic cycle.

Throughout the 14 stages of the spermatogenic cycle it has been demonstrated that the number of Sertoli cells per unit length of seminiferous tubule remains constant (Wing and Christensen, 1982). In contrast, the volume of Sertoli cells has been found to undergo marked cyclic changes. Morphometric analysis has shown that the volume of Sertoli cells is smallest during stages VII-VIII ($5300\text{-}5500\mu\text{m}^3$) and largest during XII-XIV ($7700\text{-}8000\mu\text{m}^3$) (Kerr, 1988).

Ultrastructural observations have also revealed stage-dependent variations in Sertoli cell organelles. For example, the distribution and number of secondary lysosomes have been shown to change during the cycle of the seminiferous epithelium. At most stages, the lysosomes form clusters which are located in the supranuclear region of the Sertoli cell next to the tip of the recesses which invaginate the apical cytoplasm and which contain the elongate spermatids. However, at stages VI, VII and VIII, the lysosomes accumulate at the base of the Sertoli cell cytoplasm next to the basement membrane (Morales *et al.*, 1985). Spermiation takes place at the end of stage VIII, at which time the heads and the proximal part of the tails of the

spermatozoa disengage from the Sertoli cell processes that encapsulate them. The majority of the spermatids' residual cytoplasm detaches from these cells and remains associated firmly to the luminal plasma membrane of Sertoli cells. The residual bodies are then surrounded by Sertoli cell processes, thereby forming a double-membrane phagosome which is then drawn down through the body of the Sertoli cells during stage IX of the spermatogenic cycle (Morales *et al.*, 1986). Simultaneously, the basally located lysosomes migrate towards the centre of the Sertoli cell, surrounding and merging with the phagosome. Following fusion, the inner membrane and the content of the residual bodies are rapidly lysed. Thus, in this process, residual bodies and lysosomes are eliminated from the cytoplasm.

The amount of lipid contained within the Sertoli cell also changes during the spermatogenic cycle, for it has been demonstrated that during stages IX-X, the lipid content of the Sertoli increases dramatically, coinciding with the phagocytosis of residual bodies (Niemi and Kormano, 1965; Kerr and de Kretser, 1975). It has been suggested that the lipid droplets are a by-product of the digested residual bodies. Furthermore, it has been shown that, following disturbance to the Sertoli cell by procedures such as x-irradiation, hypophysectomy and a disturbance of androgen metabolism, there is an increase in the amount of lipid in the Sertoli cell, coincident with morphological evidence of germ cell degeneration (Lynch and Scott, 1951; Lacy, 1967). However, autolysis and phagocytosis of germ cells by Sertoli cells may not be the only source of these lipid droplets, because they are known to accumulate within the Sertoli cell in the absence of germ cells (Bergh, 1981).

Stage-dependent variations in the volume of rough endoplasmic reticulum (ER) within the Sertoli cell have also been noted (Kerr, 1988). In a more recent study, Ueno and Mori, (1990) demonstrated that there was a dramatic increase in rough ER at stage VII, which consisted mostly of cisternae with a narrow lumen and with numerous ribosomes, while rough ER at other stages was frequently tubular in form and bore less ribosomes. Furthermore, the rough ER at stages VII-VIII was found in close association with mitochondria more frequently than at other stages. These findings may imply that rough ER at stages VII-VIII is involved in the active production of proteins. A study by Sharpe *et al.* (1992) has demonstrated that the incorporation of ^{35}S -methionine into secreted proteins by isolated seminiferous tubules was more than twice as great at stages VI-VIII than at stages II-V or IX-XII.

It is generally accepted that the Sertoli cell supports and nourishes developing germ cells through the many stages of differentiation and development. As the major factor which changes during the spermatogenic cycle is the complement of germ cells with which the Sertoli cell is associated, it is presumed that it is this change which triggers the morphological, and thus functional, activities of the Sertoli cell according to the specific requirements of the developing germ cells.

2.3.1.5. The blood-testis barrier

Within the testis there exists a functional blood-testis barrier which isolates germ cells at and beyond the prophase of meiosis in the adluminal compartment of the seminiferous tubule interposed between adjacent Sertoli cells and separated from spermatogonia in the basal compartment (Fawcett, 1975; Plöen and Setchell, 1992). The concept of the blood-testis barrier emerged from studies showing that acridine dyes and a wide range of other substances, when injected intravascularly, are excluded from the seminiferous tubules (de Bruyn *et al.*, 1950; Kormano, 1967, 1968). Setchell (1970a) and Waites and Setchell (1969) have also demonstrated that a number of substances of widely varying molecular size, when introduced into the bloodstream, will appear rapidly in testicular lymph, but not in the fluid collected from the cannulated rete testis. Dym and Fawcett (1970) demonstrated the existence of two functional components of the blood-testis barrier. These authors showed that when lanthanum nitrate, a small electron-opaque tracer, was introduced into the testis, the peritubular myoid cell layer excluded its passage throughout most of the length of the seminiferous tubules. In some regions, however, the tracer was able to penetrate the myoid cell layer through open junctions. At these sites the tracer also entered the interspaces between the Sertoli cells and the spermatogonia, but was prevented from penetrating further towards the tubule lumen by focal tight junctions on the interfaces between adjacent Sertoli cells. In these junctions, subsurface cisternae and bundles of filaments develop. The physiological significance of this unique junction remains unknown but it is thought to be related to the unique feature of the seminiferous epithelium, namely, the requirement for continuous upward movement of differentiating germ cells. It has been postulated that free calcium released from the endoplasmic reticulum could become bound to calmodulin, thus promoting the motile force of the microfilaments (Means *et*

al., 1980) and thereby facilitating the translocation of germ cells upwards towards the tubular lumen. According to Vitale *et al.* (1973), who used the exclusion of lanthanum, the blood-testis barrier is normally established between 16 and 19 days of age in the rat.

The main function of the blood-testis barrier is not merely the exclusion of certain large molecules, but rather the maintenance of a special ionic and hormonal environment which enables the complex process of spermatogenesis to be maintained (Waites and Gladwell, 1982). The differences in ionic composition of seminiferous tubule fluid, rete testis fluid and plasma reveal that the barrier is also capable of maintaining ionic differences within the testis, as the concentration of potassium in seminiferous tubule fluid is more than ten times higher than in plasma, while sodium and chloride concentrations are lower in seminiferous tubule fluid than in plasma (Setchell, 1980). These observations have been interpreted as providing evidence for the secretion of a potassium and bicarbonate rich fluid into the lumen of the seminiferous tubule. The passage of large molecules across the blood-testis barrier appears to be related to their lipid solubility rather than to their molecular size (Setchell, 1980). It is presumed that lipid soluble molecules enter by traversing the Sertoli cells (Setchell, 1980), and that water soluble materials enter via the spaces between the cells, only to be obstructed by the tight junctions between neighbouring Sertoli cells (Dym and Fawcett, 1975; Russell, 1978). The barrier also has a secondary functional role in that it protects the haploid germ cells from the immune system; this may be important, as surface autoantigens may be expressed for the first time following meiotic division of germ cells (O'Rand and Romrell, 1980).

2.3.1.6. Sertoli cell secretory functions

Sertoli cells are known to have a very important secretory function which includes the production of seminiferous tubule fluid (Setchell, 1980 and discussed in 2.3.1.9) and the synthesis and secretion of many hundreds of proteins which are thought not only to control spermatogenesis at the paracrine level (discussed in section 2.4), but also to modulate the functions of other intratesticular cells, including Leydig cells and peritubular cells.

2.3.1.7. Sertoli cell proteins

Over the past 20 years, the development and use of procedures such as cell isolation and cultures, 2-dimensional electrophoresis, radiolabelling

procedures, protein purification, immunolocalization and RNA analysis has led to the discovery of many Sertoli cell proteins. According to Bardin *et al.* (1988) over one hundred different proteins have been found to be secreted by Sertoli cells *in-vitro*. As very few secretory vesicles have been observed in the Sertoli cell cytoplasm either *in-vivo* or *in-vitro* it appears that, once synthesized, these proteins are probably secreted immediately (Tindall *et al.*, 1985). Within the context of this literature review those proteins which have particular significance, in the regulation of spermatogenesis, will be discussed.

Transport and binding proteins

Androgen-binding protein (ABP): ABP was the first secretory product of the Sertoli cell to be isolated and characterized biochemically. ABP is a heterodimeric glycoprotein of 85kDa, composed of two subunits with apparent molecular weights of 45kDa (H subunit) and 41kDa (L subunit), which is reported to be present in the isolated native molecule in a ratio of 3:1 (Larrea *et al.*, 1981). ABP binds testosterone and dihydrotestosterone with high affinity. ABP is secreted into the lumen of the seminiferous tubule, from where it is transported to the epididymis via the rete testis and efferent ducts (Hansson *et al.*, 1975; Bardin *et al.*, 1981). Immunohistochemical staining has demonstrated that ABP is taken up by the epithelium of the proximal portion of the caput epididymis, as minimal amounts are detectable in the lumen of the vas deferens (Pelliniemi *et al.*, 1981; Attramadal *et al.*, 1981).

A number of studies have shown that immature rat Sertoli cells in culture secrete ABP. The secretion of ABP is stimulated by FSH, cAMP and testosterone (Fritz *et al.*, 1976; Rommerts *et al.*, 1978), and the stimulation of ABP by FSH has been shown to occur in the presence of the anti-androgen cyproterone acetate (Louis and Fritz, 1979). The results of the latter study suggest that testosterone and FSH act independently on Sertoli cells to increase the secretion of ABP, and its secretion in the adult has been shown to be stage-dependent, with maximal secretion occurring at stages VII-XI of the spermatogenic cycle and minimal secretion occurring at stages II-V (Ritzén *et al.*, 1982). Similarly, the distribution of ABP mRNA, in the rat testis, has been found to be highest during stages VII-VIII (Sjögren *et al.*, 1991). Studies using antibodies to ABP have demonstrated that it is related immunologically

to testosterone binding globulin (TeBG, or sex hormone binding globulin), which is secreted by the liver in species such as man (Cheng *et al.*, 1984).

The precise function of ABP in spermatogenesis remains unknown, but the following functions have been hypothesized: 1) The fact that ABP has a high binding affinity for testosterone and dihydrotestosterone suggests that it is a transport protein within the Sertoli cell: 2) It has been demonstrated that ABP and TeBG are endocytosed by germ cells (Gérard *et al.*, 1991) which suggests that steroid binding proteins may act as steroid transporters during spermatogenesis: 3) The presence of ABP in epididymial fluid and epididymial cells, and the demonstration of receptor-mediated endocytosis of ABP in the epididymis (Guéant *et al.*, 1991) further support the hypothesis that ABP is an androgen-transporting protein.

Transferrin: Transferrin, a glycoprotein with a molecular weight of 76.5kDa, is presumed to be involved in the transport of iron. Testicular transferrin was first identified in the spent media of Sertoli cells *in-vitro* based on the molecular weight and iron-binding ability of this media and its precipitation with an antiserum against purified serum transferrin (Skinner and Griswold, 1980). Subsequent analysis of transferrin, secreted *in-vitro* by Sertoli cells, revealed that the peptide sequence was identical to serum transferrin, except that the two proteins exhibited different glycosylation patterns (Skinner *et al.*, 1984). Indirect immunofluorescence techniques have been used to determine the localization of transferrin. This protein was found to be immunolocalized in the interstitium and also to the acrosome of round and elongating spermatids (Sylvester and Griswold, 1984).

The regulation of transferrin secretion by Sertoli cells has been studied by a number of investigators. Studies using isolated Sertoli cells *in-vitro* have demonstrated that transferrin secretion can be stimulated by treating cells with insulin, FSH and retinol (Skinner and Griswold, 1982). Similarly, it has been shown that expression of transferrin mRNA by Sertoli cells *in-vitro*, treated with the same complement of vitamins and hormones parallels the increased protein production (Huggenvik *et al.*, 1987). Another study found that transferrin secretion could be increased by epidermal growth factor and by plating at higher cell densities, but was unaffected by FSH alone (Perez-Infante *et al.*, 1986).

The synthesis and secretion of transferrin has been shown to be stage-dependent. Wright *et al.* (1983) showed that transferrin secretion was maximal

at stages XII-XIV of the spermatogenic cycle and minimal at stages VI-VIII. Furthermore, *in-situ* hybridization studies have demonstrated that levels of transferrin mRNA are maximal at stages XII-XIV and minimal at stages VIII-IX (Morales *et al.*, 1987). Taken together, the results of these studies indicate that Sertoli cell transferrin mRNA and protein production are maximal during the stages in which the two divisions of meiosis occur and haploid cells are first formed, and are minimal during the stages in which translocation of preleptotene spermatocytes, spermiation and residual body phagocytosis occur.

The mechanisms by which iron is transported to developing germ cells in the seminiferous epithelium is reasonably well documented (Huggenvik *et al.*, 1984). It is presumed that iron from serum transferrin is taken up by receptor-mediated endocytosis at the basal membrane of the Sertoli cells (Morales and Clermont, 1986; Wauben-Penris *et al.*, 1986). Iron is released to the Sertoli cell and the apotransferrin is returned subsequently to the basal membrane. The iron is then incorporated into testicular transferrin, which has been synthesized by the Sertoli cell, and is secreted adluminally to pachytene spermatocytes and round spermatids, which are known to possess specific binding sites for transferrin (Holmes *et al.*, 1983; Sylvester and Griswold, 1984; Brown, 1985; Vanelli *et al.*, 1986).

Ceruloplasmin: Ceruloplasmin is a protease-sensitive serum protein with a molecular weight of 130kDa, which is considered to be the copper transport protein of serum. Ceruloplasmin is required for the cellular uptake of copper ions, which are incorporated largely into cytochrome oxidase and copper and zinc superoxide dismutase. Evidence that Sertoli cells synthesize and secrete a ceruloplasmin-like protein (testicular ceruloplasmin) was first reported by Skinner and Griswold (1983), who demonstrated that immunoprecipitation of radiolabelled proteins, secreted by Sertoli cells, with purified rat ceruloplasmin, resulted in the precipitation of a 130kDa molecular weight protein.

The cDNA for rat ceruloplasmin has been cloned and sequenced partially and probes generated from this cDNA have been shown to recognize a 3.8kb transcript in testicular RNA (Aldred *et al.*, 1987). The regulation of ceruloplasmin expression and secretion in response to hormones and growth factors has not been studied extensively. It has been shown, however, that germ cell-conditioned medium is capable of inducing ceruloplasmin RNA in

cultured rat Sertoli cells in the first two hours of exposure (Stallard and Griswold, 1990). The polarity of secretion, the immunolocalization and stage-specificity of the secretion of ceruloplasmin have still to be determined.

Sulphated glycoprotein-2 (SGP-2): SGP-2 is a glycoprotein consisting of two non-identical subunits of 34 and 47kDa, which are linked by disulphide bonds (Kissinger *et al.*, 1982; Collard and Griswold, 1987). *In-situ* hybridization, using biotinylated cRNA probes, has shown that Sertoli cells are the only cell type in the testis with SGP-2 mRNA. Immunocytochemistry has localized SGP-2 to the Sertoli cells and to the acrosome and distal tail portion of late spermatids and mature spermatozoa (Sylvester *et al.*, 1984).

The regulation of SGP-2 expression has been examined *in-vitro* and *in-vivo*. It has been shown that neither FSH nor testosterone affects SGP-2 mRNA levels in cultured rat Sertoli cells (Hugly *et al.*, 1988). However, it has been shown that hypophysectomy of young rats (aged 40 days) results in a decrease in the levels of testicular SGP-2 mRNA, which is reversed partially by FSH but not by testosterone. When 60 day old rats are hypophysectomized, SGP-2 mRNA levels are decreased similarly, and testosterone treatment is able to restore these levels partially, whereas FSH is ineffective. Hypophysectomy of adult rats (aged 90 days) results in no change in SGP-2 levels and no response to chronic testosterone replacement is observed (Roberts *et al.*, 1991).

SGP-2 is found in high concentrations in seminiferous tubule fluid and in association with the sperm surface. Recent studies in the rat have shown that the testicular form of SGP-2 dissociates from the sperm surface in the rete testis, efferent ducts and the initial segment of the epididymis where it is endocytosed (Hermo *et al.*, 1991; Sylvester *et al.*, 1991). The protein is then replaced by a slightly smaller form of SGP-2 (which is probably less heavily glycosylated) which is secreted by the epithelial cells of the caput epididymis and which attaches to the sperm surface.

Fritz and co-workers (1983) were the first to describe a potential function for SGP-2. They found a heat stable protein in ram rete testis fluid which was capable of causing suspended cells to aggregate *in-vitro* and designated the protein 'clusterin'. SGP-2 is thought to be involved in lipid transport/metabolism on the sperm cell membrane (Sylvester *et al.*, 1991). SGP-2 is similar to a serum protein which is a potent inhibitor of terminal complement complexes (O'Bryan *et al.*, 1990). The complement system is a

cascade-like defence barrier which consists of more than 20 plasma proteins that together form complement which causes the lysis of cell membranes on cells such as bacteria and viruses. In view of this, it has been suggested that SGP-2 might protect sperm against complement-mediated cell lysis in the female reproductive tract (Jenne and Tschopp, 1989).

Proteases

Cyclic Protein-2 (CP-2): CP-2 is a heterogenous glycoprotein of approximately 32.5-38kDa. Two dimensional gel electrophoresis of proteins secreted *in-vitro* by ST isolated at different stages of the spermatogenic cycle has shown that the secretion of CP-2, in the adult, is maximal at stages VI-VIII and minimal at stages XII-XIII (Wright *et al.*, 1983). Analysis of the biosynthesis of CP-2 has indicated that it is composed of a single polypeptide core which represents most, if not all, of the translation product of the CP-2 mRNA. Furthermore, the rate of biosynthesis of CP-2 was found to vary in the same stage-specific manner as its rate of secretion (Wright, 1988).

Immunohistochemical analysis has been used to determine the localization of CP-2. This protein was found to be immunolocalized only in Sertoli cells at stages V-VII of the spermatogenic cycle, (Zabludoff *et al.*, 1990a; Maguire *et al.*, 1993). CP-2 was found not be detectable by immunocytochemistry in any other tissues of the reproductive tract, although immunoblot analysis revealed the presence of CP-2 in the rete testis and epididymial fluids. Furthermore, Zabludoff *et al.* (1990b) showed the presence of CP-2 in three other organs; the proximal convoluted tubule of the kidney, the brain (with the greatest concentration in the supraoptic and paraventricular nuclei) and the posterior pituitary.

The regulation of CP-2 synthesis and secretion of CP-2 has been studied recently by Wright *et al.* (1989). These authors showed that, when mature Sertoli cells were cultured in combination with insulin, transferrin and epidermal growth factor (3H), or in combination with 3H, to which had been added FSH, testosterone, corticosterone, progesterone, vitamin E and retinol acetate, no stimulation of CP-2 secretion could be detected. These data raise the possibility that CP-2 is a unique Sertoli cell product, in that its synthesis and secretion are not regulated by hormones. However, it does appear to be regulated by particular germ cell types (Maguire *et al.*, 1993).

A partial sequence analysis of CP-2 mRNA has revealed that CP-2 is the proenzyme form of the cysteine protease, cathepsin-L (Erickson-Lawrence

et al., 1991). Northern blot analysis of testis mRNA has revealed a major (1.7kb) and minor (2.2kb) transcript with undetectable levels at stages II and XII and maximum levels at stages VI and VIIa,b. Taken together, this data suggests that CP-2/cathepsin-L gene expression is regulated in a cell-cell manner and that, in Sertoli cells, the expression of this enzyme is influenced by germ cells at different stages of development (Maguire *et al.*, 1993). The precise function of CP-2 in spermatogenesis remains unknown. It has been proposed, however, that at stages V-VII, secreted CP-2/cathepsin-L degrades adhesion molecules which bind compacted spermatids to Sertoli cells, thereby facilitating movement of these spermatids towards the lumen of the seminiferous tubule.

Plasminogen Activator (PA): Plasminogen activator is a highly specific serine protease which converts plasminogen to plasmin. This protease is known to be involved in cell migration and tissue remodelling (Reich, 1978). Sertoli cells isolated from immature rats have been shown to secrete PA in culture (Lacroix *et al.*, 1977), whilst in the adult the secretion of PA has been shown to be stage-dependent, with maximal secretion occurring at stages VII-VIII of the spermatogenic cycle (Lacroix *et al.*, 1981). These stages contain tissue-restructuring processes such as the migration of preleptotene spermatocytes from the basal compartment of the seminiferous tubule to the adluminal compartment (Russell, 1980) and release of mature spermatids from the seminiferous epithelium at stage VIII (Leblond and Clermont, 1952). Further evidence of the role of PA comes from studies by Vihko *et al.* (1984) who showed that, in the absence of preleptotene spermatocytes, the peak in PA secretion normally associated with stages VII-VIII is abolished.

Primary cultures of immature rat Sertoli cells and seminiferous tubules isolated from adult rats at different stages of the spermatogenic cycle have been shown to secrete two types of PA. When cultured under basal conditions, both Sertoli cells and seminiferous tubules produce a urokinase-type PA. When Sertoli cells and seminiferous tubules are stimulated by either FSH or dibutryl cAMP, PA secretion is increased. The PA produced under these conditions is a tissue-type PA which has a molecular weight of 70kDa (Hettle *et al.*, 1986). These two forms of PA are the products of separate genes showing approximately 50% homology (Pennica *et al.*, 1983).

Studies using cRNA probes for both urokinase-type and tissue-type PA have demonstrated that urokinase-type PA mRNA is most abundant in

stages VI-VIII, whereas tissue-type PA shows smaller variations between the different stages of the spermatogenic cycle. Both FSH and dibutryl cAMP have been shown to increase the level of tissue-type PA mRNA and production in stages VII-VIII, without affecting the levels of urokinase-type PA mRNA. In contrast, retinoic acid has been shown to increase the level of urokinase-type PA mRNA. These results show that the expression of urokinase-type PA and tissue-type PA genes is regulated differentially at specific stages of the spermatogenic cycle (Vihko *et al.*, 1989).

A recent study by Rosselli and Skinner (1992) has shown that PA production increases during pubertal development. Furthermore, these authors demonstrated that purified PModS had no effect on PA production. PModS is a paracrine factor which is produced by peritubular myoid cells and which has been shown previously to have dramatic effects on the secretion of transferrin and ABP by Sertoli cells *in-vitro* (Skinner *et al.*, 1988; Norton and Skinner, 1989; Anthony *et al.*, 1991). Thus, the finding that PA is not regulated by PModS, suggests that PModS is not a general activator of all Sertoli cell secretory functions.

Factors involved in cell growth/differentiation

Inhibin: Evidence for a non-steroidal, water-soluble testicular regulator which affects the secretion of gonadotrophins from the pituitary gland was first proposed by McCullagh (1932). Subsequent studies have demonstrated that inhibin is a gonadal peptide which can suppress specifically FSH secretion from gonadotrophs in the anterior pituitary gland (de Jong, 1979). That Sertoli cells were the site of inhibin production was first demonstrated by Steinberger and Steinberger (1976), who showed that co-culture of anterior pituitary cells and immature Sertoli cells, or the addition of Sertoli cell conditioned medium to anterior pituitary cells in culture resulted in a significant decrease in the levels of FSH in the culture medium.

The fully processed form of inhibin has a molecular weight of 32kDa and exists as a heterodimer of two dissimilar subunits, termed α and β , linked by disulphide bridges. There are two forms of the unglycosylated beta (β) subunits: β_A and β_B (Mason *et al.*, 1985), and these form two different inhibins: inhibin A and inhibin B (for reviews Ying, 1988; de Kretser and Robertson, 1989).

Sertoli cells derived from immature rats have been shown to express mRNA for the α - and β -subunits of inhibin *in-vitro* (Keinan *et al.*, 1989; Toebosch *et al.*, 1988). Immunohistochemical analysis and *in-situ* hybridization has shown the presence of mRNA for the α - and β -subunits of inhibin in the adult (River *et al.*, 1988, Roberts *et al.*, 1989). The production of inhibin in Sertoli cells is stimulated by the actions of FSH, cAMP analogues and phosphodiesterase inhibitors; this suggests that FSH stimulates inhibin production via a cAMP-dependent mechanism (Toebosch *et al.*, 1988; Bicsak *et al.*, 1987; de Kretser and Robertson, 1989).

The synthesis and secretion of inhibin has been shown to be stage-dependent, with the highest level of expression of inhibin α - and β_B mRNAs occurring in stages XIII-I, and the lowest level of expression in stages VII-VIII (Bhasin *et al.*, 1989). The secretion of immunoactive inhibin exhibits a similar pattern (for review see Parvinen, 1993). The stage-specific secretion of inhibin by isolated seminiferous tubules suggests that inhibin may have a paracrine role in testicular regulation. This hypothesis is supported by studies by van Dissel-Emiliani *et al.* (1989) who showed that inhibin administration reduced spermatogonial numbers in testes of adult mice and Chinese hamsters. These studies demonstrated that the numbers of type A₄, intermediate and type B spermatogonia were decreased significantly when compared to controls following intraperitoneal or intratesticular injections of bovine follicular fluid extracts into mice and hamsters, respectively, but that the numbers of undifferentiated spermatogonia did not change.

Activin: In addition to the $\alpha\beta$ -heterodimers of inhibin, $\beta\beta$ -homodimers, termed activins, which have a stimulatory effect on FSH release by pituitary cells *in-vivo* (Vale, 1986), have been identified. Activin shares considerable homology with transforming growth factor- β , which is also produced by the seminiferous tubule (Teerds and Dorrington, 1993). Sertoli cells contain mRNA for both the α - and β -subunits of inhibin and are thus capable of secreting activin and inhibin (Bhasin *et al.*, 1989; Grootenhuys *et al.*, 1989; Kaipia *et al.*, 1992). Recent studies on the localization of expression of mRNA for the activin receptor and site of binding of activin, have suggested that pachytene and secondary spermatocytes and step 1-5 spermatids are the target sites for activin within the seminiferous tubule (Kaipia *et al.*, 1992; de Winter *et al.*, 1992; Woodruff *et al.*, 1992). Activin has been demonstrated to increase DNA synthesis and proliferation of spermatogonia in co-cultures of

germ cells and Sertoli cells (Mather *et al.*, 1990) suggesting that activin might have a specific function in the control of germ cell development.

Interleukin-1 (IL-1): Testes of the rat and man have been shown to produce an interleukin-1 like factor with a molecular weight of about 17kDa (Khan *et al.*, 1987, 1988). Available evidence suggests that it probably originates from the Sertoli cells (Syed *et al.*, 1988), and this has now been shown definitively (Gérard *et al.*, 1991). In the rat, secretion of IL-1 by seminiferous tubules increases at puberty, coinciding with the onset of spermatogenesis (Syed *et al.*, 1988), varies according to the stage of the spermatogenic cycle and appears to correlate with spermatogonial DNA synthesis (Parvinen *et al.*, 1991; Söder *et al.*, 1991). As IL-1 is mitogenic for a variety of cell types, it is possible that the Sertoli cell might stimulate germ cell development directly, through the production of IL-1. It has been shown recently that phagocytosis of residual bodies or cytoplasm from elongate spermatids, or even the phagocytosis of latex beads, induces the secretion of IL-1 by rat Sertoli cells *in-vitro* (Gérard *et al.*, 1992). These data suggest that phagocytosis of the residual bodies at stage VIII, and their subsequent lysosomal digestion at stage X, triggers the production of IL-1 which, in turn, stimulates the first division of the differentiated (type A) spermatogonia.

Energy Metabolites

Lactate and Pyruvate: Sertoli cells metabolize glucose, via glycolysis, at a high rate, resulting in the net production of lactate and pyruvate in the ratio of approximately 4:1 (Jutte *et al.*, 1981; Robinson and Fritz, 1981). The secretion of both lactate and pyruvate can be stimulated by FSH *in-vitro* (Jutte *et al.*, 1982, 1983; Mita *et al.*, 1982). In studies which have compared the effects of glucose and lactate upon the viability and function of spermatocytes and spermatids, it has been demonstrated that these cells cannot maintain their ATP levels if supplied with glucose, but can do so on addition of lactate and, to a lesser extent, pyruvate (Jutte *et al.*, 1981; Mita and Hall, 1982; Grootegoed *et al.*, 1984). Furthermore, it has been shown that spermatocytes and round and elongating spermatids contain a testis-specific isozyme of lactate dehydrogenase (LDH-C4). This enzyme has an equilibrium biased towards the production of pyruvate and, as such, catalyses preferentially the oxidation

of lactate to pyruvate (Meistrich *et al.*, 1977). Thus, it would appear that Sertoli cells supply spermatocytes and spermatids with energy, in the form of lactate, which is oxidized subsequently to pyruvate for use in the tricarboxylic acid cycle.

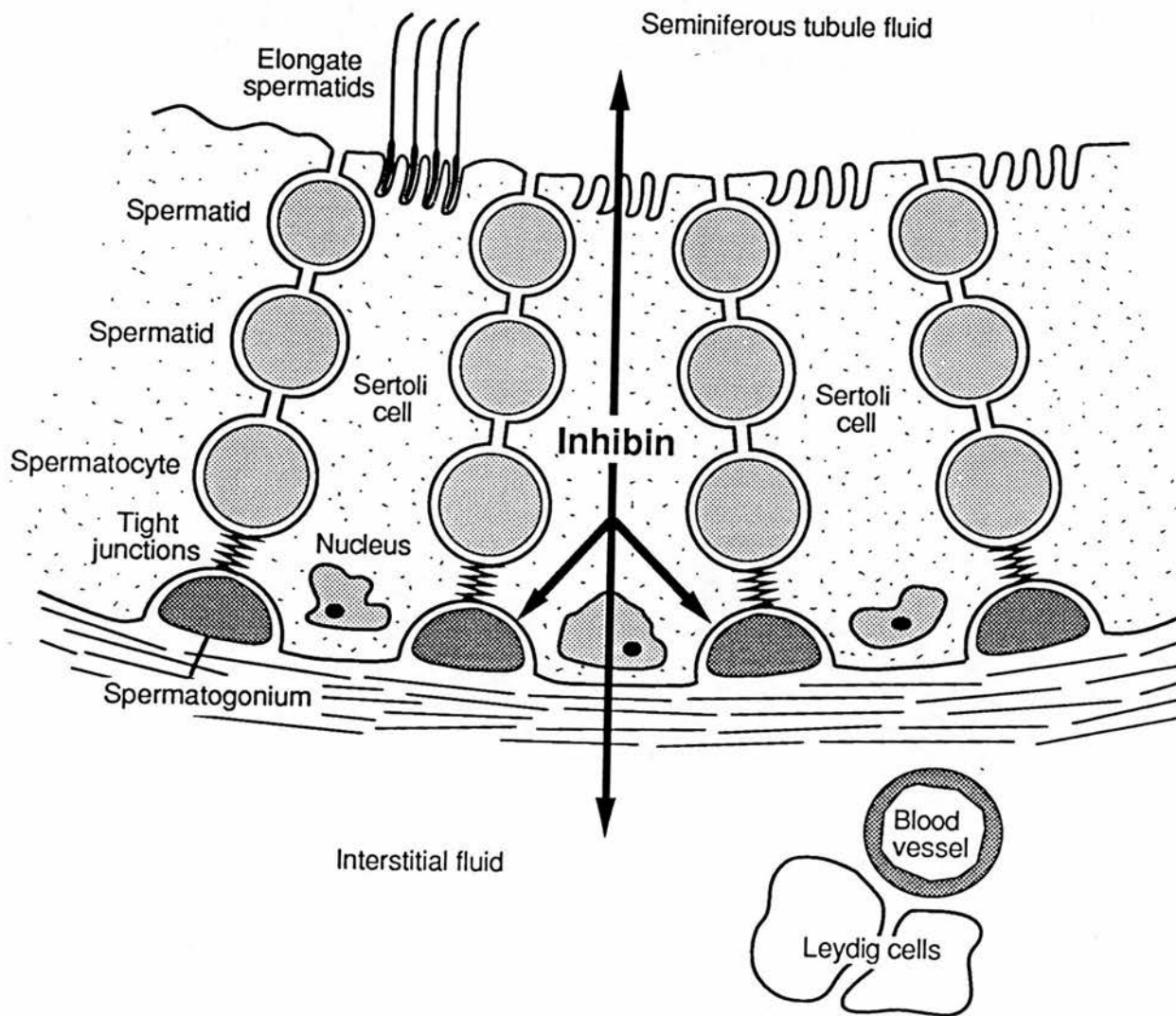
α -Keto acids: In the rat seminiferous epithelium, branched-chain aliphatic amino acids can be converted to the corresponding α -keto acid by the enzyme aminotransferase, which is present in Sertoli cells (Grootegeod *et al.*, 1985) but not germ cells. Such α -keto acids are converted subsequently to α -hydroxy acids by the testis specific enzyme, lactate dehydrogenase (LDH-C4) which, it may be suggested, also shuttles NAD/H into germ cells so as to facilitate lactate utilization for energy.

2.3.1.8. Bidirectional secretion by the Sertoli cells

It is well recognized that Sertoli cell proteins are secreted in a bidirectional manner (Sharpe, 1988) i.e. via the base of the Sertoli cell into testicular interstitial fluid (IF) and thence into the bloodstream, or via the apex of the Sertoli cell into seminiferous tubule fluid (STF) (Figure 2.5). Several recent findings suggest that bidirectional secretion by the Sertoli cell may be of physiological importance.

It has been shown that, in the adult rat, 80% of ABP secreted by the Sertoli cell is secreted apically and is then taken up by the epididymis. This could imply that the remaining 20% of ABP, which is secreted via the base of the Sertoli cell, may be due to 'leakage' and is unimportant (Gunsalus *et al.*, 1980; Mather *et al.*, 1983). However, recent studies suggest that this is not the case. Firstly, regardless of whether it is measured in peripheral blood or in testicular IF, the secretion of ABP via the base of the Sertoli cell declines progressively during puberty in the rat (Gunsalus *et al.*, 1980; Nazian, 1986; Sharpe and Bartlett, 1987). This pattern of decline is clearly not related to the formation of the inter-Sertoli cell tight junctions at around 18 days of age (Dym and Fawcett, 1970). Secondly, when spermatogenesis is disrupted following treatment with busulphan or ethane dimethane sulphonate, exposure to short-term local testicular heating or cryptorchidism, or following hypophysectomy, then ABP secretion into testicular IF is increased substantially (Gunsalus *et al.*, 1980, 1981; Bartlett and Sharpe, 1987; Morris *et al.*, 1987, 1988; Sharpe and Bartlett, 1987). In contrast, in many of these

Figure 2.5. Schematic diagram of the epithelium of part of the seminiferous tubule to illustrate the association of germ cells in various stages of development (spermatogonia, spermatocytes, spermatids) with the supporting Sertoli cells. The latter co-ordinate germ cell development via the secretion of specific proteins (here typified by inhibin) which are secreted or 'leak', into STF in the lumen of the tubule and into IF which surrounds the tubule. Either of these routes may result in the appearance of testicular proteins in blood. (Courtesy of R. Sharpe).



situations the secretion of ABP into the tubule lumen is decreased, as is the overall production of ABP (Jégou *et al.*, 1983; Jégou *et al.*, 1984; Morris *et al.*, 1987, 1988). Furthermore, when spermatogenesis is disrupted specifically, resulting in the loss of a particular germ cell type, altered ABP secretion can be related to the absence of specific germ cell type (i.e. pachytene spermatocytes and elongate spermatids) (Pinon-Lataillade *et al.*, 1986; Morris *et al.*, 1987; Bartlett *et al.*, 1988). Taken together, these results suggest that the overall secretion of ABP, in addition to the proportion that is secreted via the apex and base of the Sertoli cell, is modulated by the complement of germ cells *in-vivo*. In support of this thinking, *in-vitro* studies have shown that the addition of isolated germ cells or germ cell-conditioned medium to Sertoli cells in culture can also modulate the secretion of ABP (Galdieri *et al.*, 1984; Le Magueresse *et al.*, 1986; Le Magueresse and Jégou, 1986).

Other Sertoli cell products which have been shown to be secreted bidirectionally include inhibin (Maddocks and Sharpe, 1989a, 1990; Allenby *et al.*, 1991a), testibumin (Cheng and Bardin, 1986) and, probably, transferrin (Janecki and Steinberger, 1987a).

2.3.1.9. Seminiferous tubule fluid production

The secretion of seminiferous tubule fluid is an important function of Sertoli cells; it has been suggested that this fluid is essential for the nutrition of germ cells and the transport of seminiferous tubule secretory products from the basal to the apical portion of the seminiferous epithelium. It is also required for the release and transport of spermatozoa to the epididymis (for review see Jégou, 1992).

Studies by Jégou *et al.* (1982) using efferent duct ligation, to assess the production of seminiferous tubule fluid in the rat, demonstrated that fluid production begins at about 20 days of age, when lumen formation is first evident (Vitale *et al.*, 1973). At about the same time, inter-Sertoli cell junctions are first established, meiotic activity is first seen among germ cells and the seminiferous tubules increase in diameter and length. Thus, it seems likely that fluid production in the seminiferous tubule is dependent upon the development of several morphological features.

The composition of tubule fluid has been reviewed by Waites and Gladwell (1982) and Setchell and Brooks (1988). The most characteristic findings are very high concentrations of potassium and bicarbonate, and low levels of sodium and chloride, in comparison to blood plasma. Another

difference between this fluid and plasma is the extremely low total concentration of proteins in the tubule fluid, which illustrates the efficiency of the blood-testis barrier in preventing blood-borne products entering freely into the seminiferous tubules.

Until quite recently it was generally believed that the regulation of seminiferous tubule fluid production was not under the direct control of gonadotrophins (Setchell, 1978). However, there are several pieces of evidence suggesting that fluid production is under hormonal control. It has been shown that, following hypophysectomy, there is a decrease in fluid production in the rat (Setchell, 1970), whilst in earlier experiments, Murphy (1965a,b) reported 'secretory hypertrophy' of the Sertoli cell and an increase in tubule diameter, which might be attributable to increased fluid production. Direct evidence for the hormonal control of tubule fluid production was provided by Jégou *et al.* (1983), who demonstrated that, in the immature rat, only FSH, and not testosterone propionate, LH, hCG or prolactin, was able to stimulate fluid production whereas, in the adult rat, fluid production was found to be controlled principally by testosterone. In fact, seminiferous tubule fluid production has been shown to decrease following testosterone withdrawal. Using efferent duct ligation, O'Leary *et al.* (1987) showed that seminiferous tubule fluid production is reduced by nearly half at seven days following EDS treatment.

It has been suggested that seminiferous tubule lumen size is determined by the rate of production of seminiferous tubule fluid (Waites and Gladwell, 1982). In this respect, production of seminiferous tubule fluid is greatest at stages VI-VIII (the androgen dependent stages) of the spermatogenic cycle, based on the large increase in diameter of the seminiferous tubule lumen which has been shown to occur at these same stages (Sharpe, 1989b). This increase in lumen diameter has been shown previously to be dependent on the presence of elongate spermatids in the seminiferous epithelium (Sharpe, 1989b). Furthermore, these germ cells have been shown to exert a positive influence on the rate of production of seminiferous tubule fluid (Jégou *et al.*, 1984).

2.3.2. Interstitial tissue

The interstitial tissue, found between seminiferous tubules, constitutes about 16% of the total testis volume in the rat, and contains Leydig cells, endothelial cells, lymphatic vessels, macrophages and blood vessels (Christensen, 1975;

Kerr, 1989). The Leydig cells form nearly 3% of the total volume of the rat testis, and morphometric analysis has shown that there are approximately 22 million Leydig cells per gram of tissue (Mori and Christensen, 1980). The interstitium of the testis provides a unique environment in which Leydig cells synthesize and secrete testosterone into the vascular system and seminiferous tubule compartment.

2.3.2.1. The Leydig Cell

Mammalian Leydig cells are relatively large polyhedral cells. At the EM level, Leydig cells are characterized generally by a round or ovoid nucleus, prominent mitochondria (which are found throughout the cytoplasm), abundant smooth endoplasmic reticulum (ER) (which forms a continuous network throughout the cytoplasm) and scattered patches of rough ER (which interconnect with the smooth ER). The Golgi complex is well developed and often found at one pole of the nucleus. The cytoplasm also contains lipid droplets, lysosomes, microtubules and microfilaments. Some of these organelles may play prominent roles in Leydig cell steroidogenic function. Briefly, lipid droplets contain cholesterol esters and neutral fats which can be released into the cytoplasm, where they are hydrolyzed by soluble esters to provide a source of cholesterol in testosterone biosynthesis (Christensen, 1975; Ewing and Zirkin, 1983). Mitochondria contain the enzyme complex responsible for the conversion of cholesterol to pregnenolone, whilst the smooth ER contains a family of enzymes which convert pregnenolone to testosterone. In common with other steroid secretory cells, the Leydig cells possess extensive smooth ER, and it has been demonstrated that a positive correlation exists between testosterone production and the amount of smooth ER in Leydig cell cytoplasm (Zirkin *et al.*, 1980). In contrast, these authors were unable to demonstrate a positive correlation between testosterone and the amount of inner and outer mitochondrial membrane, rough ER and lipid droplets.

2.3.2.2. Leydig cell steroidogenesis

Testosterone synthesis results from the effect of multiple enzymes acting in a cascade sequence on cholesterol. The latter can be derived from three separate sources. The principal source is *de novo* synthesis from acetate, which occurs in the smooth ER. A second source is the Leydig cells which contain numerous lipid droplets containing cholesterol esters which can be

hydrolyzed to cholesterol by plasma esterases. A third source is blood plasma from which cholesterol can be obtained via a cholesterol-lipoprotein-cell membrane receptor system (Ewing and Zirkin, 1983; Rommerts and van der Molen, 1989).

The initial and rate limiting step in the synthesis of testosterone is catalyzed by the cytochrome P450-dependent cholesterol side chain cleavage enzyme, cytochrome P450_{scc}. P450_{scc} is a mitochondrial enzyme which catalyses the conversion of cholesterol to pregnenolone by removal of the 6-carbon side chain on the C-17 of cholesterol. Testosterone can be converted from pregnenolone through two separate pathways (Δ^4 and Δ^5) in the smooth ER. In the Δ^4 pathway pregnenolone is converted to testosterone via intermediates, progesterone, 17 α -hydroxyprogesterone and androstenedione by the actions of 3 β -hydroxysteroid dehydrogenase, 17 α -hydroxylase, C17-20 lyase and 17 β -dehydrogenase, respectively whereas, in the Δ^5 pathway, pregnenolone is converted to testosterone via 17 α -hydroxypregnenolone, dehydro-epiandrosterone and androstenediol (Rommerts and van der Molen, 1989). The testicular rate of conversion of pregnenolone through the Δ^4 and Δ^5 pathways is different in different animal species. In the rat testis, the Δ^4 pathway appears to be the most important whereas, in the human testis, the Δ^5 pathway appears to be the most significant.

A number of recent studies have utilized the cytotoxic effects of ethane dimethane sulphonate (EDS) in order to investigate the role of testosterone in spermatogenesis. A single intraperitoneal injection of EDS disturbs selectively Leydig cell function (Morris and McClukie, 1979) resulting in the complete destruction of all Leydig cells within 36 hours (Kerr *et al.*, 1985; Morris *et al.*, 1986). This leads to undetectable levels of testosterone within the testis within 24 to 36 hours, resulting in the selective degeneration of pachytene spermatocytes, followed by round spermatids, restricted initially to stage VII of the spermatogenic cycle (Bartlett *et al.*, 1986; Sharpe *et al.*, 1990). Germ cell degeneration can be prevented by administering regular exogenous doses of long acting testosterone esters every three days (Sharpe *et al.*, 1988a,b, 1990). Such treatment can maintain testicular weight, sperm output and fertility for 8 to 10 weeks (Sharpe *et al.*, 1988b). Furthermore, when testosterone is withdrawn from adult rats as a result of treatments such as immunoneutralization of LH (Dym and Raj, 1977), hypophysectomy (Russell and Clermont, 1977) or by a variety of steroidal agents known to reduce gonadotrophin secretion severely (Russell *et al.*, 1981), the earliest morphological change is the degeneration of pachytene spermatocytes and

step 7 and 19 spermatids at stage VII. These observations demonstrate that testosterone acts specifically at stage VII and this, together with the growing awareness that germ cells are able to modulate the secretory function of the Sertoli cell, implies that the mechanisms by which testosterone controls spermatogenesis via the Sertoli cell are dependent on the presence of a particular complement of germ cells. Since the secretory function of Sertoli cells has been shown to vary in accordance with the stage of the spermatogenic cycle (Parvinen, 1982, 1993), it is presumed that testosterone must drive spermatogenesis via effects on the secretion of specific proteins by Sertoli (or peritubular) cells around stage VII. In this respect, Sharpe *et al.* (1992) have demonstrated recently that testosterone regulates both the overall level of protein secretion and the secretion of at least seven specific proteins (androgen regulated proteins) by seminiferous tubules at stage VII of the spermatogenic cycle. Moreover, these androgen-regulated events are influenced by the resident germ cells (McKinnell and Sharpe, 1992; Sharpe *et al.*, 1993). These studies, using methoxyacetic acid (a germ cell specific toxicant) to assess the effect of depletion of the germ cell types at stages VI-VIII, have shown that depletion of pachytene spermatocytes, round spermatids or elongate spermatids prevents the normal stage-dependent increase in overall protein secretion as well as affecting the secretion of the androgen regulated proteins. The identity and function of the androgen regulated proteins secreted by seminiferous tubules at stages VI-VIII has yet to be established.

2.3.2.3. Macrophages

Macrophages are known to be present within the testis of a number of species. Testicular macrophages in the rat may comprise up to 25% of the interstitial cell population (Niemi *et al.*, 1986) and are found frequently in close association with Leydig cells. The importance of this association is unclear, although Bergh (1985b) indicated that the morphology of the two cell types is correlated, so that a functional inter-relationship is likely. Yee and Hutson (1985) have demonstrated that conditioned medium from cultures of rat macrophages stimulated basal and LH-stimulated testosterone production when added to Leydig cells *in-vitro*. Macrophages secrete interleukin-1 β , which may have an important role in regulating testosterone production by Leydig cells in the adult (Calkins *et al.*, 1988) and immature rat (Verhoeven *et al.*, 1988). Studies have shown that interleukin-1 β can reduce hCG-

stimulated testosterone production and cAMP formation dramatically in whole testis cultures prepared from neonatal rats or enriched Leydig cell cultures prepared from immature or adult rats. IL-1 β was found to act by inhibiting LH receptor binding, and therefore cAMP formation, as well as inhibiting induction of P450scc mRNA levels by hCG (Lin *et al.*, 1991). These results indicate that testicular macrophages play a role in regulating testicular function.

2.4. Paracrine control of the testis

The mammalian testis is a highly complex tissue containing numerous cell types. The process of spermatogenesis takes place in the seminiferous tubules, which contain Sertoli cells and the various generations of developing germ cells. Surrounding the seminiferous tubule is a basal lamina comprising two acellular layers and a layer of peritubular myoid cells. In the interstitium of the testis, between the seminiferous tubules, are the Leydig cells which are the site of androgen production. In order to function effectively and efficiently, the testis and, indeed, any organ or tissue, must co-ordinate the activities of all its different cell types. There is now substantial evidence suggesting that the development and subsequent functioning of the different testicular cell types is co-ordinated by local (paracrine) control mechanisms.

2.4.1. Sertoli cell-germ cell interactions

As has been discussed previously, Sertoli cells exhibit both functional and morphological changes during the seminiferous epithelium cycle (for reviews see Jégou, 1992; Parvinen, 1993). Because the number of Sertoli cells per unit length of the seminiferous tubule remains constant throughout the stages of the spermatogenic cycle (Wing and Christensen, 1982) it is presumed that the cyclic morphological and functional variations in Sertoli cells are the result of the changing number and needs of the germ cell complement at each stage of the spermatogenic cycle (Jégou, 1992).

Direct evidence pointing to the existence of complex interactions between the Sertoli and germ cells comes from experimental studies which have examined the effects of selective germ cell depletion *in-vivo* on Sertoli cell secretory function *in-vitro*. Germ cell depletion can be induced by a variety of methods, including short-term local testicular heating, irradiation or chemical treatments such as meta-dinitrobenzene or methoxyacetic acid.

Additional evidence has come from *in-vitro* studies in which Sertoli cells, isolated from immature rats, have been co-cultured with either different germ cell types or germ cell-conditioned media.

***In-vitro* studies**

The first piece of evidence to show that the presence of germ cells can modify Sertoli cell secretory function was drawn from observations of the method by which germ-cell free Sertoli cells are obtained. Following the removal of germ cells, by hypotonic shock treatment, it was discovered that the secretion of several Sertoli cell proteins, including ABP, transferrin and immunoactive inhibin, was reduced significantly (Galdieri *et al.*, 1984; Le Magueresse and Jégou, 1988; Le Magueresse *et al.*, 1988, Castellón *et al.*, 1989a; Pineau *et al.*, 1990). These changes were related to the loss of germ cells rather than to aspecific effects of the hypotonic treatment, as the effects are in fact reversible when the germ cells are added back to the Sertoli cells.

The action of germ cells on Sertoli cell secretory function can vary according to the type of germ cell being tested. In this respect, pachytene spermatocytes, when added to Sertoli cell cultures, have been demonstrated to stimulate the secretion of ABP and transferrin (Le Magueresse *et al.*, 1986; Le Magueresse and Jégou, 1988a,b; Castellón *et al.*, 1989a,b) and to inhibit the production of oestradiol (Le Magueresse and Jégou, 1988a,b). Whereas, Galdieri *et al.* (1984) did not find any effect on ABP secretion when early spermatids were co-cultured with Sertoli cells, other studies have demonstrated that these cells can exert a stimulatory influence on this marker of Sertoli cell function (Le Magueresse *et al.*, 1986). Since these early studies round spermatids, in co-culture with Sertoli cells, have also been shown to inhibit the conversion of testosterone to oestradiol (Le Magueresse and Jégou, 1988a,b), to stimulate transferrin secretion (Le Magueresse *et al.*, 1988) and to enhance dramatically both the secretion of immunoactive and bioactive inhibin and the level of mRNA for the α -subunit of inhibin (Pineau *et al.*, 1990).

The effects of pachytene spermatocytes and round spermatids on Sertoli cell function are presumed to be mediated through several different, but complementary, pathways. These include cell surface molecules that mediate cell-cell adhesion (CAMs), plasma membrane structural devices such as those involved in maintenance of the cytoarchitecture of the seminiferous epithelium (for example, ectoplasmic specializations, desmosome-like

junctions), and gap-junctions, which are known to be involved in the passage of small molecules between one cell type and another and the production of several soluble factors.

That germ cells could produce factors which regulate Sertoli cell function was first proposed by Le Magueresse and Jégou (1986), who demonstrated that media conditioned by a mixed population of germ cells, obtained from the testes of adult rats, contained heat and trypsin-sensitive factor(s) that were able to stimulate and inhibit ABP and oestradiol secretion, respectively. Subsequently, Ireland and Welsh (1987) found that such spent media could stimulate the phosphorylation of several Sertoli cell proteins.

These early observations have now been extended by recent studies which have used bicameral chambers for the culture of Sertoli cells. These chambers enable the Sertoli cells to form confluent epithelial sheets that, by virtue of the Sertoli cell tight junctions, form transepithelial permeability barriers between the apical and basal compartments of the chamber (Janecki and Steinberger, 1987a; Djakiew and Dym, 1988; Onoda and Djakiew, 1990, 1991). These studies have shown that the addition of pachytene spermatocytes or round spermatids, or media conditioned by these cells, alters the predominant route of secretion of various proteins, with the majority of proteins being secreted apically rather than basally. Thus, it appears that germ cells are able to influence the composition of their surrounding milieu by altering Sertoli cell secretory function, and this provides further support for the concept of paracrine interactions between Sertoli and germ cells during spermatogenesis.

Pachytene spermatocytes and round spermatids may also affect Sertoli cell function by modulating gene expression. Fujisawa *et al.* (1992) found that, when immature rat Sertoli cells were co-cultured in the presence of either pachytene spermatocytes or round spermatids, the expression of the opioid precursor gene preproenkephalin (PPenk), found in Sertoli cells, was increased 6.4 and 1.9-fold.

***In-vivo* studies**

The use of Sertoli cells isolated from immature rats, which have only been exposed to pachytene spermatocytes and round spermatids *in-vivo*, have one distinct disadvantage, in that there is increasing evidence suggesting that, at each step of testicular development, the regulation of Sertoli cell activity is

taken over essentially by the most advanced generation(s) of germ cells present within the seminiferous epithelium (Jégou *et al.*, 1988; Jégou, 1991). Thus, an alternative approach is to use adult rats which have been exposed to a variety of treatments known to induce the selective loss of a particular germ cell type, in order to assess their specific role in regulating adult Sertoli cell function.

Short-term local testicular heating is known to induce the loss of primary spermatocytes and early spermatids which, by a maturation depletion process, results in a progressive reduction in the number of elongating and elongate spermatids (Chowdhury and Steinberger, 1970). Jégou *et al.* (1984) demonstrated that, under these circumstances, Sertoli cell activity decreased, paralleling closely the reduction in the number of late spermatids, as evidenced by reduced ABP and seminiferous tubule fluid production and increased plasma FSH levels. This observation is largely independent of the degree of loss of earlier germ cell types and is the same whether germ cell depletion is induced by treatment with busulphan (Morris *et al.*, 1987) or irradiation (Pinon-Lataillade *et al.*, 1988; Pineau *et al.*, 1989). On the basis of these results it appears that, in the adult rat, the presence of late (elongating and elongate) spermatids may be a prerequisite for normal Sertoli cell function.

Further evidence showing that elongate spermatids can modulate Sertoli cell secretory function was provided by studies in which spermatogenesis was disrupted by treatment with the germ cell specific toxicant, methoxyacetic acid (MAA). Allenby *et al.* (1991a) demonstrated that elongate spermatids may control positively the secretion of immunoactive inhibin by the Sertoli cell, based upon the finding that its level in plasma was reduced by nearly 50% when elongate spermatids were grossly depleted, and returned progressively to normal as these cells repopulated the seminiferous epithelium. Furthermore, when seminiferous tubules were isolated, from rats in which elongate spermatids had been depleted by treatment with MAA 3-4 weeks earlier, their ability to secrete immunoactive inhibin *in-vitro* was also reduced by 50% or more.

In another study, in which germ cell depletion was induced in adult rats by treatment with MAA, the diameter of the seminiferous tubule lumen, which normally doubles in size at stages VII and VIII of the spermatogenic cycle, failed to increase at these stages at three weeks after treatment, coincident with the depletion of elongate spermatids. The absence of earlier germ cell types was without effect (Sharpe, 1989b). The absence of elongate

spermatids is also associated with a marked increase in the secretion of interstitial fluid volume, the production of which is thought to be controlled by Sertoli cells (Maddocks and Sharpe, 1989).

The mechanisms underlying the control of Sertoli cell function by late spermatids have still to be identified. There is currently no evidence demonstrating that late spermatid gene products are secreted (Hecht, 1988). There are, however, three other potential routes whereby late spermatids might influence Sertoli cell function (Jégou, 1991; Jégou *et al.*, 1992). Firstly, alterations in the type of adhesive contact between late spermatids and Sertoli cells may influence signals between the two cell types. For example, the calcium-dependent cell adhesion molecule, N-cadherin, stimulates dramatically neurite outgrowth (Doherty *et al.*, 1991). Interestingly, N-cadherins are also present in the testis and are expressed in Sertoli cells and spermatids (Byers *et al.*, 1993). A second possible route involves a direct cytoskeletal link between the late spermatid-Sertoli cell interface and the nuclear transcription machinery so that externally-imposed shape changes, caused by cell substratum or cell-cell contact, could influence gene transcription directly. The third possible route involves the residual bodies, which are the structures shed from late spermatids during spermiation. When Sertoli cells phagocytose spermatid residual bodies they are ingesting as much as 70% of the total spermatid cytoplasm (Russell, 1980). It has been hypothesized that residual bodies may regulate Sertoli cell function via mRNA coding for factor(s) which control Sertoli cell gene expression; Sertoli cell factors may then regulate different events at the germ cell level.

That other germ cell types can also contribute to the control of Sertoli cell function in the adult testis has been shown by Vihko *et al.* (1984). Following the depletion, by X-irradiation, of one of the four or five layers of germ cells that are normally associated with the Sertoli cell, the secretion of plasminogen activator was found to be abolished only after the selective depletion of preleptotene spermatocytes.

Whilst it is obvious that Sertoli cell function is affected by other cell types in the testis, there is little doubt that the most profound and important interactions are between the Sertoli cells and developing germ cells, firstly, because multiplication, meiosis and translocation of the differentiating germ cells have to occur at precise time intervals, secondly, because all of the basic metabolic requirements of the meiotic and post-meiotic germ cells have to be supplied by Sertoli cells, (for example, lactate, transferrin) and thirdly,

because the metabolic requirements of germ cells change according to the stage of their development.

2.4.2 Sertoli cell-peritubular cell interactions

The stromal cells that surround the seminiferous tubule and are in contact with the basal surface of the Sertoli cells are referred to as the peritubular myoid cells (for reviews see Skinner, 1991; Verhoeven, 1992). Peritubular cells are thought to provide structural support for the tubule and contraction of the tubule (Clermont, 1958; Ross and Long, 1966), and they are constituents of the blood testis barrier (Dym and Fawcett, 1970; Plöen and Setchel, 1992). Together with Sertoli cells, the peritubular myoid cells produce an extracellular matrix (ECM). The ECM separates the Sertoli and peritubular cells and forms the basement membrane of the tubule (Skinner and Fritz, 1985a; Skinner *et al.*, 1985; Hadley and Dym, 1987).

It has been shown recently that the ECM is an essential requirement for cell migration, proliferation and cell polarity. The presence of an ECM in culture is known to promote a histotype of Sertoli cells which appears similar to that found *in-vivo*, with a columnar shape cell, a nucleus that is located near the basal surface of the cell and tight junctions between Sertoli cells (Tung and Fritz, 1984; Anthony and Skinner, 1989). The observation that the presence of the ECM promotes a Sertoli cell with a 'normal' appearance led several laboratories to develop dual-chamber culture systems in order to investigate polarized secretion by Sertoli cells. The presence of an extracellular matrix was found to facilitate the polarization of the cell and create a permeable barrier (Byers *et al.*, 1986). Furthermore, peritubular cells were found to increase the efficiency of the permeability barrier and to alter the polarized secretion of several Sertoli cell proteins (Janecki and Steinberger, 1987b; Ailenberg and Fritz, 1989; Ailenberg *et al.*, 1988).

Peritubular cells have also been shown to co-operate metabolically with immature Sertoli cells in culture (Hutson, 1983), and have been demonstrated to stimulate the production of Sertoli cell total protein (Hadley *et al.*, 1985), of ABP production, in both FSH-stimulated and untreated Sertoli cells (Tung and Fritz, 1980; Hutson and Stocco, 1981) and transferrin (Holmes *et al.*, 1984; Hadley *et al.*, 1985). These cells were shown subsequently to produce a non-mitogenic factor that modulates Sertoli cell factor (Skinner and Fritz, 1985b, 1986). This factor, termed PModS, has been shown to stimulate the production of a number of Sertoli cell proteins, including ABP, transferrin

and inhibin (Skinner *et al.*, 1988, 1989), as well as augmenting the actions of androgens on Sertoli cell function (Skinner and Fritz, 1985c). It has been suggested that LH acts on Leydig cells to stimulate androgen production which, in turn, acts on peritubular cells to regulate PModS production which, in turn, can act on Sertoli cells to modulate functions associated with the maintenance and control of germ cell differentiation and development. The potential importance of PModS in the maintenance of spermatogenesis and testicular function led Skinner *et al.* (1988) recently to isolate from peritubular cell conditioned medium, two proteins with PModS activity. These proteins, termed PModS A and PModS B, differ in apparent molecular weight (56 and 59 kDa, respectively). Both forms of the protein have been demonstrated to mimic all the mentioned stimulatory effects of PModS on Sertoli cells and their effects are accompanied by increased production of cGMP (Norton and Skinner, 1989). The relationship between PModS A and PModS B is, however, unknown at present.

The actions of peritubular cells on Sertoli cell morphology and secretory function is relatively well known. In contrast, the way in which Sertoli cells interact, and thus influence peritubular cell function, remains relatively unknown. The theory that alterations in Sertoli cell function may affect peritubular cell function is based on the finding that perturbation of Sertoli cell function, coincident with the depletion of late spermatids, results in the alteration of the peritubular tissue (Pinon-Lataillade *et al.*, 1988). In a more recent study, it has been proposed that germ cells may be able to modulate peritubular cell function indirectly, via a complex interaction including testosterone, pachytene spermatocytes, round spermatids, Sertoli and peritubular cells (McKinnell and Sharpe, 1992).

2.4.3. Sertoli cell-Leydig cell interactions

The dependence of spermatogenesis on normal Leydig cell function, notably normal testosterone secretion, is now well established. There is, however, substantial evidence that Sertoli cells exert a paracrine influence over Leydig cell development, number and function (for review see Sharpe, 1990, 1993).

The first morphological evidence indicating a local effect of seminiferous tubules on Leydig cells came from a study by Moore in 1924a, who reported that cryptorchidism resulted in Leydig cell 'hyperplasia' adjacent to damaged seminiferous tubules. More recently, Aoki and Fawcett (1978) induced focal atrophy of seminiferous tubules in the testis, by the

implantation of silastic capsules containing cyproterone acetate, and found a dramatic hypertrophy of the interstitial tissue adjacent to the damaged tubules. In addition, electron micrographs of Leydig cells in these regions exhibited a large increase in the amount of smooth endoplasmic reticulum. It was concluded by Aoki and Fawcett that the changes in the volume and ultrastructure of Leydig cells could not be due to an imbalance in the normal feedback to the hypothalamo-pituitary axis, as any hormonal stimulation would cause a general stimulatory response of the Leydig cells, and not a local response confined to the immediate vicinity of the damaged tubules. Accordingly, it was speculated that some form of local control mechanism was involved.

The existence of local control mechanisms between Sertoli and Leydig cells has also been postulated, on the basis of a number of observations which suggest that Leydig cells may undergo cyclic changes, for it has been demonstrated in the rat that Leydig cells lying adjacent to seminiferous tubules at stages VI-VIII of the spermatogenic cycle are significantly larger than those adjacent to tubules at other stages (Berg, 1983, 1985a). A subsequent study by Fouquet (1987) failed to confirm this difference in Leydig cell size in either rats or monkeys, but found that the volume of smooth endoplasmic reticulum in Leydig cells adjacent to stage VII tubules, in the rat, was higher than in Leydig cells adjacent to seminiferous tubules at other stages. As it is well established that the volume of SER correlates positively with the capacity of Leydig cells to secrete testosterone (Ewing and Zirkin, 1983), this finding is still consistent with stage VII tubules regulating the levels of testosterone in their immediate vicinity.

Direct evidence that Sertoli cells can modulate Leydig cell function has come from many studies which have shown that the co-culture of Sertoli and Leydig cells or the addition of Sertoli cell-conditioned medium (SCCM) to Leydig cells *in-vitro* results in increased basal and LH(hCG)-stimulated testosterone production. In the rat, such studies have used isolated Sertoli and Leydig cells, both from immature rats (Verhoeven and Cailleau, 1985, 1986, 1990) or Sertoli cells from immature rats and Leydig cells from pubertal (Janecki *et al.*, 1985) or from adult rats (Onoda *et al.*, 1991). More recently, conditioned medium prepared from cultures of adult human Sertoli cells has also been shown to stimulate basal and hCG-stimulated testosterone production by isolated human Leydig cells as well as by MA-10 mouse tumour cells (Papadopoulos, 1991). These results suggest that one or more paracrine factors, secreted by Sertoli cells into the interstitial fluid

surrounding the seminiferous tubules, act on the neighbouring Leydig cells, causing an increase in testosterone production. In this respect it has been demonstrated that testicular IF, from the rat, contains one or more nonsteroidal factors which enhance Leydig cell testosterone production *in-vitro*, especially LH/hCG-stimulated testosterone production (Sharpe and Cooper, 1984). Furthermore, it has been shown that, when spermatogenesis is disrupted by procedures such as cryptorchidism (Sharpe and Cooper, 1984; Sharpe *et al.*, 1986; Risbridger *et al.*, 1987), local testicular heating (Bartlett and Sharpe, 1987) or treatment with EDS (Sharpe *et al.*, 1986; Drummond *et al.*, 1988), the level of this stimulatory factor(s) in IF is increased.

Several investigators have attempted to characterize the Sertoli factors which stimulates Leydig cell steroidogenesis. This factor has been shown by Verhoeven and Cailleau (1986, 1987) to be present in both human and rat Sertoli cell-conditioned medium and to have a molecular weight of 10-30kDa. Human Sertoli cells have also been shown to secrete a stimulatory factor with a molecular weight of 79kDa, which is different from transferrin, human serum albumin and testibumin (Papadopoulos *et al.*, 1991). In the rat, the Leydig cell stimulatory factor has been shown to be secreted via the base of the Sertoli cell and to be modulated specifically by pachytene spermatocytes, but not by round spermatids (Onoda *et al.*, 1991).

In contrast to the general agreement of studies of the effects of isolated Sertoli cells or Sertoli cell-conditioned medium on Leydig cell function, the effects of isolated seminiferous tubules from adult rats on Leydig cell function is highly variable. Stimulatory, as well as inhibitory, effects have been reported, depending not only on the stage of the seminiferous tubules, but also on the purity of the cultures and the age of the rats from which the Leydig cells have been isolated. For example, co-cultures of seminiferous tubules with Leydig cells have been shown to enhance basal and LH/hCG-stimulated testosterone production (Verhoeven and Cailleau, 1986; Papadopoulos *et al.*, 1987b), or to have no effect on basal testosterone production but to inhibit hCG-stimulated testosterone production (Syed *et al.*, 1988). Even more confusing is the finding that if crude, rather than purified, Leydig cells are used, inhibition of testosterone production by seminiferous tubule in co-culture or seminiferous tubule-conditioned medium is found (Parvinen *et al.*, 1984; Syed *et al.*, 1985). One explanation for the disparity between findings in studies which have used either ST-Leydig cell co-cultures and studies which have used ST-conditioned medium, is that the former may



be able to metabolize some of the testosterone produced by Leydig cells, the result of which would be to obscure any stimulatory effects (Qureshi, 1993).

Several Sertoli cell secretory products have been identified that can modulate Leydig cell function, for example GnRH and arginine vasopressin (AVP). GnRH receptors within the rat testis are located only on the Leydig cell (Bourne *et al.*, 1980; Sharpe *et al.*, 1982), and the addition of GnRH or its agonists to isolated Leydig cells has short term stimulatory effects on basal testosterone production both *in-vitro* (Sharpe and Cooper, 1982) and *in-vivo* (Sharpe *et al.*, 1983). On prolonged exposure the effects of GnRH become inhibitory (Browning *et al.*, 1983). A GnRH-like peptide has been reported within the testis (Sharpe *et al.*, 1981) and this peptide is thought generally to interact with Leydig cell receptors. Attempts to purify testicular GnRH have revealed that it is different from hypothalamic GnRH and that more than one active component may exist (Bhasin *et al.*, 1983). To date, testicular GnRH has not been isolated, possibly due to conflicting reports about the presence (Popkin *et al.*, 1985) and the absence (Clayton and Huhtaniemi, 1982) of GnRH receptors. The ability of GnRH to modulate the testicular response to LH stimulation, and its ability to alter testicular blood flow, and the possibility that it may act over short periods of time, all suggest, however, that testicular GnRH may play a role in local regulation of testosterone production in the rat testis.

There is also substantial evidence that arginine vasopressin (AVP) might play a paracrine role in the regulation of Leydig cell function. Studies in the rat and in the mouse have demonstrated that there are receptors for AVP on isolated Leydig cells (Meidan and Hsueh, 1985; Tahri-Joutei and Pointis *et al.*, 1988). Furthermore, the presence of an AVP-like substance has been demonstrated in rat testis extracts (Kasson *et al.*, 1985) and in testicular IF (Pomerantz *et al.*, 1988). The addition of AVP to isolated rat Leydig cells has been shown to have short-term stimulatory effects on testosterone production (Sharpe and Cooper, 1987), which are inhibitory in the long-term (Tahri-Joutei and Pontis *et al.*, 1988). However, the extremely low level of expression of AVP mRNA in the testis (Ivell, 1992) casts some doubt on the physiological role of AVP in the paracrine control of Sertoli cell-Leydig cell interactions.

Sertoli cells also produce a number of growth factors that may play a role in mediating Sertoli-Leydig cell interactions (for reviews see Bellvé and Zheng, 1989; Skinner, 1991; Mather and Krummen, 1992). One of these is transforming growth factor- β (TGF- β), which belongs to a family of growth

factors which includes inhibin, activin and mullerian-inhibiting substance (MIS). TGF- β , when added to primary cultures of rat Leydig cells, has been shown to reduce the number of LH receptors, without affecting the binding affinity of the ligand (Avallet *et al.*, 1987). In addition, TGF- β has been reported to inhibit hCG-stimulated testosterone production in rat Leydig cell cultures, but may also stimulate some aspects of basal steroidogenesis, as shown by the enhanced conversion of pregnenolone to testosterone. TGF- β binding has been demonstrated on porcine Leydig cells (Avallet *et al.*, 1987). Thus, it may be suggested that TGF- β may be involved in modulation of the gonadotrophin responsiveness of Leydig cells.

3. General Materials and Methods

This chapter describes materials and methods common to a number of studies in this thesis. Methods unique to specific experiments are described in the relevant chapters.

3.1. Animals

The animals used in all experiments were either male Wistar rats bred in the MRC Reproductive Biology Unit in Edinburgh, or Sprague-Dawley rats obtained from Charles River (UK). Rats were housed, under conventional controlled conditions (12 hours light: 12 hours dark cycle), at a temperature of 21°C with food and water available *ad libitum*. Animals were killed by inhalation with 100% carbon dioxide followed by cervical dislocation.

3.2. Seminiferous tubule isolation procedure

Seminiferous tubules (ST) were isolated and cultured from the testes of control and treated 70-80 day old Wistar rats using slight modifications of the method of Allenby *et al.* (1991a,b). Testes were kept on ice, decapsulated within 2 hours of removal from the animal and placed into a small plastic Petri dish containing ice cold Dulbecco's phosphate buffered saline (PBS; Flow labs, Irvine, Scotland) on a cooled (4°C) transparent Perspex stage illuminated from below. Lengths of seminiferous tubules were then isolated by teasing apart gently the central portion of the testis using fine watchmakers' forceps. Tubules were isolated in lengths >2cm (approximate range 2-5cm). Isolated tubules were transferred to a separate Petri dish containing fresh PBS. Long lengths of tubules were used as it has been shown previously that this is optimal for the maintenance of Sertoli cell inhibin secretion and hormonal responsiveness and, more importantly, secretion of inhibin by the isolated tubules reflect accurately changes induced experimentally *in-vivo* (Allenby *et al.*, 1991a,b). In these studies, seminiferous tubules were isolated with an intact peritubular cell layer so as to maintain, as far as possible, the cytoarchitecture and cell-cell interactions which would be found normally *in-vivo* (Sharpe *et al.*, 1990). Once sufficient lengths of tubules had been isolated they were transferred to a separate Petri dish containing fresh PBS and dissected into stages II-V, VI-VIII or IX-XII of the spermatogenic cycle, according to the criteria of Parvinen (1982). This relies on differences in the transilluminated appearance of the ST at each stage as a

consequence of changes in the density and position of the heads of the elongate spermatids. Selection of stage groupings was based both on the ease with which particular stages can be distinguished from one another and on the stage-specificity of the early adverse effects which local heating and testicular toxicants (described in the relevant chapters) have on spermatogenesis *in-vivo*. ST were cut using a scalpel and only those ST lengths >0.5cm were used. ST which showed signs of stretching or damage were excluded. Staged ST were transferred subsequently to fresh PBS and, by reference to a transparent grid, a total of 10cm ST at each stage grouping was transferred to a 24-well plastic culture plate (Nunc, Kampstrup, Denmark) containing 0.1ml culture medium; this medium consisted of Eagles minimal essential medium without methionine (Flow Labs) containing 4mmol/L L-glutamine (Sigma, Dorset, England), 100mU penicillin/ml, 100µg streptomycin/ml (Flow Labs), 25mmol/L Hepes (Gibco, Paisley, Scotland) and 0.1% polyvinyl alcohol (PVA; Sigma) per well. PVA was used as a protein substitute. Immediately prior to incubation, 60µCi ³⁵S-labelled methionine (Specific Activity >1000 Ci/mmol: ICN Flow, High Wycombe, England) was added to each culture. ST were cultured for 24 or 72 hours at 32°C in a humidified atmosphere of 5% carbon dioxide and 95% air. Each 10cm ST was incubated in a final volume of 0.4ml. At the end of incubation, the medium was aspirated from the ST into an Eppendorf tube containing a protease inhibitor (Aprotinin: final concentration 5% (v/v): Sigma). The medium was then centrifuged at 13,000 rpm for 5 minutes and the aspirate stored at -40°C. ST remaining in the well were washed three times with 0.5ml PBS and lysed subsequently by the addition of 250µl 0.5mmol/L Tris-HCl and freeze-dried at -40°C. Preliminary studies have shown this method results in the recovery of >95% of intracellular ³⁵S-methionine labelled proteins.

3.3. Analysis of radiolabelled proteins

3.3.1. Evaluation of newly synthesized proteins by TCA precipitation

Incorporation of ³⁵S-methionine into secreted proteins (from either ST culture medium or Sertoli cell+germ cell co-cultures) or intracellular proteins (from ST lysates) was determined by precipitation with 10% (v/v) trichloroacetic acid (TCA) using the following procedure:

Three separate 10µl aliquots of samples were incubated on ice for 15-30 minutes in the presence of 10µl 0.1M PBS (containing 10mg/ml BSA (Sigma), and 1ml 10% (v/v) TCA (containing 10mM/L-methionine). Samples were

then centrifuged at 13,000 rpm for 5 minutes and the resulting supernatant removed carefully and discarded. The pellet remaining was washed with 200µl 0.1M potassium hydroxide, resuspended in 1ml 10% (v/v) TCA, vortexed and left on ice for a further 15-30 minutes before being recentrifuged at 13,000 rpm for 5 minutes. Following aspiration of the supernatant the pellet was dissolved in 200µl 0.4M potassium hydroxide and vortexed. The ^{35}S -methionine content of the precipitate was counted in an NEN (New England Nuclear, USA) beta counter. Incorporation for each individual sample was computed as the mean of the three replicates run for each sample.

3.3.2. Isoelectric focussing (first dimension separation)

All procedures utilized a water-cooled Protean II electrophoresis system and a model 3000 xi power supply from Bio-Rad Laboratories (Hemel Hempstead, England). The method used was a modification (Sharpe *et al.*, 1992) of the technique described by O'Farrell (1975). Gels for isoelectric focussing consisted of 5.5g Urea (Sigma), 1.33ml 30% acrylamide/BIS (Bio-Rad Labs), 2ml 10% nonidet P-40 (BDH, Poole, Dorset, England), 0.6ml ampholines (0.4ml pH 5-7, 0.15ml pH 7-9, 0.05ml pH 3-10 - Bio-Rad Labs) and 2.15ml deionised distilled water (DD-water). After degassing the monomer solution for 2 minutes, polymerization was initiated by the addition of 20µl 10% (w/v) fresh ammonium persulphate and 10µl TEMED (N,N,N,N'-tetramethylethylenediamine) (both from Sigma). Isoelectric focussing gels were poured to a height of 11cm in 160mm x 2.5mm internal diameter glass capillary tubes (Bio-Rad Labs). Gels were then overlaid with 50µl DD-water and left for approximately 2 hours to polymerize. At this time the overlay was replaced with 20mM NaOH and left for a further hour. Before mounting the gels on the electrophoresis unit, the NaOH at the top of the gels was replaced with degassed NaOH. Care was taken to remove air bubbles from the ends of the tube gels. The gels were prefocussed at 200V for 15 minutes, 300V for 30 minutes, and 400V for 15 minutes, using 6mmol/L phosphoric acid as the anolyte and freshly degassed 20mmol/L NaOH as the catholyte.

Samples were prepared for loading by the addition of an equal volume of isoelectric focussing sample buffer (IEF) (9.5mol/L urea, 2% nonidet P-40, 2% ampholines, 1% dithiothreitol) and additional urea was added to ensure a final concentration of 9.5mol/L. The samples were incubated at room temperature for 15 minutes before being centrifuged at 13,000 rpm for 3 minutes and the supernatant loaded on to the tube gels. Each tube gel was

loaded with equal counts per minute (cpm) ^{35}S -methionine labelled proteins (200-250,000cpm). In order to measure the pH gradient created by the ampholines, blank tube gels were loaded with 50 μl IEF sample buffer. Gels were then prefocussed at 400V for 14 hours followed by 800V for 2 hours. Sample gels were then extruded from the glass tubes using water pressure and incubated in a storage tube which contained 0.5ml sample buffer (made up of 10ml 0.5mol/L Tris HCL, pH 6.8, 20ml 10% sodium dodecyl sulphate, 4ml 0.05% bromophenol blue (Bio-Rad Labs) and 36ml DD water) for 3 minutes at room temperature before being frozen rapidly in ethanol: dry ice and stored at -40°C until required. Blank tube gels were sectioned into 24 x 5mm segments, each of which was placed into 1.4mls DD-water, and allowed to stand at room temperature for 4-6 hours, before measurement of the pH.

3.3.3. Second dimension separation

Second dimension gel electrophoresis (protein separation on the basis of molecular weight) utilized slab gels which contained 10% (v/v) acrylamide. Each 10% gel consisted of 13.3ml acrylamide in 10ml 1.5mol/L Tris-HCL (pH 8.8) and 16.7ml DD-Water. After degassing for 15 minutes, polymerization was initiated by the addition of 600 μl fresh 10% (w/v) ammonium persulphate and 18 μl TEMED. Once poured, the gels were overlaid with water-saturated iso-butanol (Aldrich Chemical Co., Dorset, UK), and left for approximately 1 hour to polymerize. The butanol was removed carefully and the top of the gel washed thoroughly with clean water. If the gels were for immediate use they were overlaid with separating gel buffer (0.375 mol/L Tris-HCl, pH 8.8) for a further 2-3 hours. If the gels were for use the next day, then the separating gel buffer was left in place, the top of the gel case was sealed and the unit stored at 4°C overnight. Frozen IEF gels were thawed at room temperature and then transferred carefully to the top of the slab gel to ensure that no air bubbles were trapped; no sealing medium was used. Molecular weight markers (phosphorylase b, 97kDa; albumin, 67kDa; carbonic anhydrase, 43kDa; trypsin inhibitor, 20.1kDa; α -lactalbumin, 14.4kDa; Sigma) were loaded. The electrolyte buffer used contained 0.3% Tris base, 1.44% glycine and 0.1% sodium dodecyl sulphate (all from Sigma) in DD-Water. Gels were run at 38mA/gel (constant current) for approximately 2 hours and silver stained as described in Section 3.3.4.

3.3.4. Silver staining

Gels were fixed in 40% (v/v) methanol : 10% (v/v) acetic acid for a minimum of 30 minutes, followed by 10% (v/v) ethanol : 5% (v/v) acetic acid for a further 30 minutes. The gels were then silver stained using the Bio-Rad silver staining kit (Bio-Rad Labs). Gels were first washed in an oxidizing solution for 5 minutes and then washed in clean water for 30 minutes. Gels were then placed in silver staining solution for 20 minutes followed by a short rinse of 1 minute in clean water. Developer was then added until a smokey precipitate was observed; this precipitate was then discarded and replaced with fresh developer for 5 minutes. The developing solution was then again replaced with fresh developer which was left in contact with the gels for approximately 20 minutes, or until such time when the gels appeared to be optimally stained. The developing action was then stopped by the addition of 5% (v/v) acetic acid, and the gels either stored in clean water until required or dried immediately. Prior to drying, gels were soaked for 15 minutes in Amplify (Amersham International, Amersham, England), and dried under vacuum for 2 hours at 62°C on a model 543 drier (Bio-Rad Labs).

3.3.5. Development of autoradiographs

Dried gels were exposed to x-ray film (Kodak X-OMAT ARS) at -80°C for 21 days. Before development, autoradiographs were allowed to warm to room temperature, at which time the film was placed in developer (Kodak) for 3 minutes, washed thoroughly with tap water, fixed (Kodak fixative) for 1 minute, washed again and allowed to air dry.

3.4. Histology

3.4.1. Perfusion - fixation

The perfusions were performed using techniques described previously by Kerr *et al.* (1984). Rats were anaesthetized using ether; the abdominal cavity was then opened and the descending aorta located and cannulated approximately 1.0cm below the heart and the cannula tied in place with silk. The right auricle was punctured to allow exit of the perfusate. 0.9% (w/v) saline containing 5U/ml heparin was then perfused by gravity feed (3 foot height) via the cannula at a flow rate of 20mls/min until the testicular blood vessels were seen to have cleared of blood. At this time animals were perfused with fixative solution (0.17M cacodylate buffer, 2%

paraformaldehyde, 0.26M picric acid, plus 3% glutaraldehyde, pH 7.4 approximately 500mOSM) at a flow rate of 6-8mls/min for 35-45 minutes until the testes were fixed. The testes were then dissected from the animal, decapsulated and the equatorial region cut into 2mm thick transverse slices using a new razor blade. These were then trimmed to a square shape and diced into cube-shaped blocks of approximately 2x2x2mm. These blocks were then placed into the same fixative solution, as used for perfusion, for 2-48 hours at 4°C before primary processing as described below.

3.4.2. Processing of perfusion-fixed testicular tissues

Tissue blocks were washed several times in 0.2M cacodylate buffer for a minimum of 4 hours and then post-fixed in 2% osmium tetroxide (in 0.2M cacodylate) for 2-3 hours at 4°C. The cacodylate washes were repeated several times for at least 30 minutes, before two further 15 minute washes in 0.05M maleate buffer. Tissue blocks were then post-fixed in 1% (w/v) uranyl acetate for 2 hours at room temperature and rewashed in maleate buffer for 2 periods of 15 minutes. After this the blocks were washed twice in increasing concentrations of ethanol (70,80,90,95%) (v/v) for 10 minutes each, then washed several times in 100% ethanol for 1 hour. They were then washed twice in neat propylene oxide for 30 minutes, before being pre-embedded in a 50:50 mixture (v/v) of propylene oxide: epon araldite (Bio-Rad Labs) for 24 hours. After this the 50:50 mixture was poured off and the blocks placed in an oven at 60°C for 10 minutes to evaporate excess propylene oxide. The blocks were then transferred to liquid araldite for at least 6 hours and then into fresh liquid araldite for a minimum of 12 hours. Following this the blocks were embedded in fresh liquid araldite in flat-bottomed, cylindrical plastic capsules. These were placed in an oven at 60°C for 24-36 hours to allow the araldite to polymerize.

3.4.3. Sectioning

Following the removal of excess araldite from around the edges of the testicular blocks, semi-thin sections were cut to a thickness of 0.5 - 0.75µm, using glass or diamond knives, on a Reichert Jung microtome (Model No. 2050).

3.4.4. Staining

Sections of testicular tissue were stained using toluidine blue for a few seconds at approximately 60°C. The toluidine blue was always freshly filtered before use.

3.4.5. Microscopy and photography

Sections were examined and photographed using a Zeiss Photomicroscope (Zeiss, Welwyn Garden City, England).

3.5. Collection of body fluids for inhibin measurement

3.5.1. Collection of testicular interstitial fluid (IF)

Testicular IF was collected from control and treated rats using the technique described previously by Sharpe and Cooper, (1983). Rats were killed rapidly by inhalation of carbon dioxide followed by cervical dislocation. An incision was made into the scrotal sac, the testis dissected free of the epididymis and connective tissue, blotted to remove excess blood, and a small incision made in the caudal end of the testicular capsule. The testis was then placed into a pre-weighed polystyrene tube such that it was suspended 1-2cm above the tube bottom and re-weighed (to determine testicular weight). Testicular IF was allowed to drain from the testis for approximately 16 hours at 4°C. After removal of the testis the tube was centrifuged at 1000g for 5 minutes at 4°C to precipitate contaminating erythrocytes and the IF was then aspirated in measured amounts. An aliquot of IF was diluted 1:10 (v/v) with 0.1M PBS + 1% BSA and stored at -20°C before being assayed for inhibin. The 'drip' collection method used to measure IF volume is relatively crude, but it has been shown to provide a reliable index of the total volume of IF in the testis (Sharpe and Cooper, 1983).

3.5.2. Collection of plasma

Under ether anaesthesia a midline abdominal incision was made, and venous blood collected from the posterior vena cava using a heparinized syringe and needle; the animal was then killed by cervical dislocation. Blood samples were placed into heparinized tubes on ice and centrifuged at 3800g for 30 minutes at 4°C and the resulting plasma stored at -20°C for subsequent inhibin measurement.

3.6. Measurement of immunoactive inhibin

3.6.1. Iodination of inhibin tracer

Synthetic $1\text{-}^{26}\alpha$ -porcine inhibin plus glycine²⁷ tyrosine²⁸ was synthesized and provided kindly by Dr J Rivier of the Salk Institute and iodinated by the Chloramine-T method (Hunter and Greenwood, 1962). In brief, 5 μ l Na ^{125}I (500 μ Ci, Amersham International, Aylesbury, Buckinghamshire) was added to 1 μ g of $1\text{-}^{26}\alpha$ -porcine inhibin and the iodination initiated by the addition of 10 μ l fresh chloramine-T solution (1mg/ml in 0.5M PBS; BDH). The mixture was then vortexed and the reaction allowed to proceed for 1 minute before being terminated by the addition of 10 μ l sodium metabisulphate solution (1mg/ml in 0.05M PBS; BDH).

3.6.2. Tracer purification

Free ^{125}I was separated from the iodinated peptide on a 30cm column of Sephadex G-25 (Pharmacia, Uppsala, Sweden) using phosphate buffer (0.05 M: pH 7.5) containing 0.5g/L CHAPS ((3-[3-Cholamidopropyl dimethylammonio]-1-propanesulfonate)). A mini-assay gamma counter (Type 6-20, Mini Instruments, Essex) was used to count the fractions. Fractions forming the peak of activity corresponding to ^{125}I -labelled $1\text{-}^{26}\alpha$ -inhibin were pooled (see Figure 3.1). This was stored at -20°C for up to 1 month.

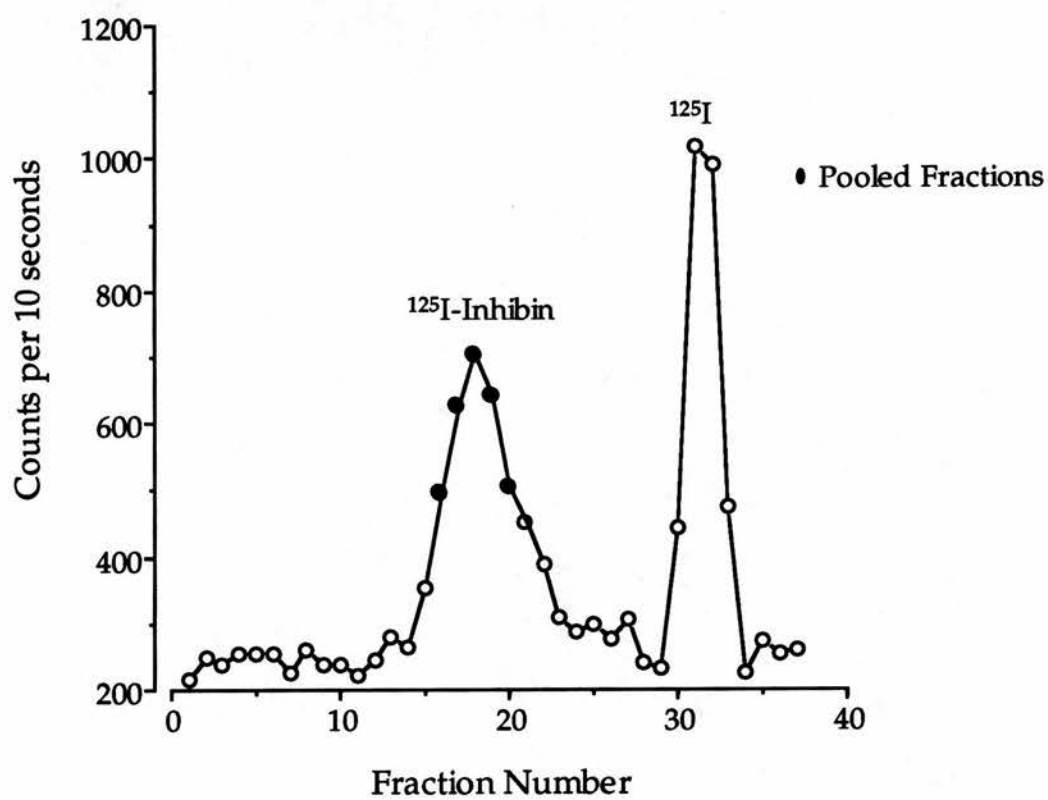
3.6.3. Inhibin standards

Standard curves were set up in each assay using a $1\text{-}^{26}\alpha$ -inhibin stock solution ($1\text{-}^{26}\alpha$ -inhibin plus glycine²⁷ tyrosine²⁸). The concentrations of standards ranged from 1000 pg/0.1ml double diluted in 0.1M PBS + 1% (w/v) BSA (crude grade) and 0.01% (w/v) thiomersalate to give standards of 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9, and 1.95 pg/0.1ml. Standards were stored at -20°C .

3.6.4. α -Inhibin radioimmunoassay procedure

Levels of α -inhibin in plasma and testicular interstitial fluid (IF) from control and treated rats were measured using a radioimmunoassay described and validated previously by Sharpe *et al.* (1988). This assay is based on an antibody (S55) raised in a sheep to the 1-26 amino acid sequence of the N-terminus of the α -subunit of porcine 32 kDa inhibin. Samples, standards

Figure 3.1. Radioactivity in fractions eluted from Sephadex G-25 column after radioiodination of $1\text{-}^{26}\alpha$ -inhibin, showing incorporation of ^{125}I into the first (protein) peak. The fractions that were pooled and used as ^{125}I -labelled $1\text{-}^{26}\alpha$ -inhibin tracer are shown also.



and quality controls were dispersed and diluted as appropriate using a programmable electronic station (Hamilton Microlab M, Hamilton Bonaduz AG, Switzerland). 100µl aliquots of standards, samples (40µl for IF) and quality controls (high and low quality controls) were incubated together with 100µl of a 1:20,000 dilution of the first antibody (S55) and 200µl 0.1M PBS + 1% BSA (w/v). After vortexing, the tubes were incubated at 4°C for 24 hours. Next, 100µl ¹²⁵I-labelled porcine inhibin, which had been diluted with assay buffer to yield approximately 15,000 cpm/100µl of solution, was added to each tube. Also included were tubes containing either 400µl 0.1M PBS + 1% BSA (NSB) or 300µl 0.1M PBS + 1% BSA plus 100µl S55 (B₀). NSB tubes were used to measure non-specific binding of the tracer to the assay tubes, whilst the B₀ tubes determined the maximal binding of the tracer. Tubes containing 100µl tracer only were used to determine the total amount of radioactivity added to each tube (total counts; TC). After a further 24 hours, 100µl normal sheep serum (Scottish Antibody Production Unit) was added to each tube at a dilution of 1:600 in 0.1M PBS + 1% BSA together with the second antibody, donkey anti-sheep serum (Scottish Antibody Production Unit), at a final dilution of 1:10. Following incubation for a further 24 hours at 4°C, 1ml 0.9% (w/v) saline was added to all tubes (except TCs) and the tubes were then centrifuged at 4°C for 30 minutes at 2500g. The supernatant was then decanted and the tubes allowed to drain. The precipitate was counted for 60 seconds in a gamma counter (NE 1600-Nuclear Enterprises). During counting the output from the Multigamma counter was written to a digital logger (Datagrabber, Mutek, Box, Wiltshire). This allowed subsequent input to an assay calculation program written by Dr Phil Taylor for the Apple Macintosh computer (AssayZap, Elsevier, Biosoft, Cambridge). Because the antibody used in this assay is raised to a peptide sequence on the α-subunit of inhibin, it can potentially also detect free α-subunit or extended forms of the α-subunit in addition to the inhibin αβ heterodimer. In practice, this appears not to be a problem as, in most instances, levels of immunoactive inhibin measured by RIA in plasma, IF, Sertoli cell- and ST-conditioned medium, and rete testis fluid have been confirmed using an *in-vitro* inhibin bioassay that uses sheep pituitary cells (Sharpe *et al.*, 1988; Maddocks and Sharpe, 1989; Maddocks and Sharpe, 1990; Pineau *et al.*, 1990).

3.6.5. Assay sensitivity

Assay sensitivity was approximately 2pg ¹⁻²⁶α-inhibin, and the inter- and intra-assay coefficients of variation were less than 11%.

3.7. Data analysis

Experimental results were analysed by analysis of variance (ANOVA) and Student's *t* test and in general have been expressed as the mean \pm SD.

4. Effect of age on ST protein secretion

The majority of *in-vitro* studies have used Sertoli cells which have been isolated from immature rats (mostly 15-18 days of age) and, as the morphological and biochemical characteristics of the Sertoli cell are known to change with age (see Chapter 1), an extrapolation from immature to adult animals cannot always be assumed. The studies described in this chapter assessed whether there are major differences in the secretion of proteins by isolated seminiferous tubules from rats of different ages and whether the protein response to the addition of known testicular toxicants is comparable at the different ages.

4.1. Introduction

It is generally believed that the Sertoli cell plays an important role in the regulation of germ cell development through a complex series of cell-cell interactions between the Sertoli cell and the developing germ cells (Jégou, 1991; Sharpe, 1993). It is presumed that these interactions are mediated by the secretion of different proteins by the Sertoli cells and that these vary cyclically according to the complement of germ cells associated with the Sertoli cell (i.e. the stage of the spermatogenic cycle) (Parvinen, 1982; 1993). In addition, there is increasing evidence to suggest that germ cells are capable of modulating the secretory function of the Sertoli cell according to their own specific requirements. This evidence derives, firstly, from studies of the consequences of germ cell depletion *in-vivo*, caused by γ irradiation (Pinon-Lataillade *et al.*, 1988), exposure to testicular toxicants (Bartlett *et al.*, 1988; Allenby *et al.*, 1991a) and, *in-vitro*, following the removal of germ cells (Galdieri *et al.*, 1981; Le Magueresse *et al.*, 1986; Le Magueresse and Jégou, 1986). Secondly, the addition of isolated germ cells or germ cell-conditioned medium to Sertoli cells in culture has been shown to alter the secretion of several Sertoli cell proteins, including androgen-binding protein, and there is general agreement that pachytene spermatocytes are more potent in this respect than are round spermatids or residual bodies (Galdieri *et al.*, 1984; Le Magueresse and Jégou, 1986; Castellón *et al.*, 1989). Similarly the secretion of inhibin (Pineau *et al.*, 1990) and transferrin has been shown to be augmented *in-vitro* by the addition of crude germ cells, or preparations enriched in either pachytene spermatocytes or round spermatids (Djakiew and Dym, 1988; Le Magueresse *et al.*, 1988). Furthermore, when medium conditioned by either pachytene spermatocytes or round spermatids is added to Sertoli cells in

bicameral chambers they cause a 2- to 3-fold increase in the overall level of protein secretion, as judged by the level of incorporation of ^{35}S -methionine (Djakiew and Dym, 1988; Onoda and Djakiew, 1990, 1991). With few exceptions, all of the *in-vitro* studies have used cultures of Sertoli cell monolayers from immature rats and, as the characteristics of these cells are known to change with age (Castellón *et al.*, 1989; Jégou, 1991), it is not certain that the results obtained in these studies are directly relevant to the adult animal (Russell and Steinberger, 1989). Furthermore, most of these studies have used Sertoli cells which have been co-cultured with a particular class of germ cell and, although particular germ cells can modify particular functions of immature Sertoli cells *in-vitro*, the presence of other germ cell types (and other cell types) found normally in the testis may serve to further modify Sertoli cell function.

The experiments described in this chapter were undertaken to assess the effect of age on seminiferous tubule protein secretion and the susceptibility of protein secretion to the adverse effects of testicular toxicants in the rat. The compounds used in these studies were meta-dinitrobenzene (m-DNB) (Foster *et al.*, 1986; Blackburn *et al.*, 1988; Allenby, 1990 and see Chapter 5) and nitrobenzene (NB) (Bond *et al.*, 1981; Levin *et al.*, 1988; Allenby *et al.*, 1990 and see Chapter 6), both of which have been shown to act on the Sertoli cell; and methoxyacetic acid (MAA) which is a well established germ cell-specific toxicant, thought to act directly on pachytene spermatocytes, (Creasy *et al.*, 1985; Bartlett *et al.*, 1988 and see Chapter 7).

4.2. Experimental Procedures

4.2.1. Seminiferous tubule ST cultures

ST were isolated from immature (aged 28 days), late pubertal (aged 45 days) and young adult (aged 70 days) rats and cultured according to the methods described in Chapter 3. As it is impossible to distinguish the various stages of the spermatogenic cycle at 28 days of age using normal criteria, which rely on changes in the density and position of the heads of elongate spermatids (Parvinen, 1982), a pool of unstaged ST was isolated from rats in the three age groups.

4.2.2. Sertoli cell+germ cell co-cultures (SC+GC)

Crude Sertoli cell+germ cell co-cultures were prepared from the testes of rats aged 28 days using the method of Gray and Beamand (1984) as modified by Williams and Foster (1988). Testes were removed and decapsulated in Hanks Basic Salt solution (HBSS) without calcium, magnesium or phenol red (Flow Labs). The testicular tissue was chopped coarsely and incubated in 0.25% (w/v) trypsin solution (Flow Labs) containing 10µg/ml DNAase (Sigma) at 32°C for 8-15 minutes. After passage through an 11 micron nylon mesh filter, the retained tissue was washed with approximately 100-150ml HBSS. The ST were then resuspended in 50ml 0.1% (w/v) collagenase (Type 1, Sigma) in HBSS and incubated at 32°C for approximately 8 minutes. The resulting solution was filtered through a 75 micron nylon mesh filter and the retained tissue washed with 100-150ml HBSS. Any remaining tissue was recovered further from the filter by backwashing with Eagles minimum essential medium, supplemented with 0.1mM non-essential amino acids, 4.0mmol/L L-glutamine (Flow Labs) (Sigma), 50µU penicillin/streptomycin/ml (Flow Labs) and 10% foetal bovine serum (v/v). Aliquots of the cellular suspension, containing small clumps of Sertoli cells and germ cells (mainly pachytene spermatocytes and round spermatids), were plated at a density of 8×10^6 cells/2.5ml/ (35mm) well. Cultures were incubated at 32°C in a humidified atmosphere of 5% carbon dioxide and 95% air for 24 hours. Thereafter, the culture medium was replaced daily with serum-free medium. At 72 hours after the initial plating, the culture medium was replaced with Eagles minimal essential medium without methionine (Flow Labs) containing 4.0mmol/L L-glutamine (Sigma); 50µU penicillin/streptomycin/ml (Flow Labs), 0.1% (PVA; Sigma); the PVA was used as a protein substitute and 500µCi ³⁵S-labelled methionine per well (Specific activity >1000 Ci/mmol : ICN Flow). Sertoli cell+germ cell co-cultures were then incubated for a further 24 hours at 32°C. At the end of incubation, the culture medium was aspirated into an Eppendorf tube containing a protease inhibitor (Aprotinin : final concentration 5% (v/v) : Sigma), centrifuged at 13,000 rpm for 5 minutes and the aspirate stored at -40°C. Incorporation of ³⁵S-methionine into secreted proteins (in the aspirated culture medium) was assessed after precipitation with trichloroacetic acid and scintillation counting. All experiments were repeated three times.

4.2.3. Sertoli cell only cultures

Sertoli cell only cultures were obtained from Sertoli cell+germ cell co-cultures (SC+GC) co-cultures as described by Galdieri *et al.* (1981), as it was found that >90% pure Sertoli cell cultures could be produced routinely by this method. Briefly, to remove the germ cells, Sertoli cell+germ cell co-cultures were exposed to hypotonic Tris (hydroxymethyl) amino methane-hydrochloride (29mM, pH 7.4) for 5 minutes, 48 hours after the initial plating. The Tris was then removed and replaced with fresh culture medium and, following a further 24 hour incubation, cultures were treated with toxicants or FSH for a further 24 hours. At the end of incubation, the medium was removed from the cultures prior to measurement of cell exfoliation.

4.2.4. Treatment of cultures

In order to assess whether there were age-dependent differences in response to adverse effects (with testicular toxicants) or hormonal effects, cultures were exposed to meta-dinitrobenzene (m-DNB), nitrobenzene (NB), methoxyacetic acid (MAA) or follicle stimulating hormone (FSH). The m-DNB (Aldrich Chemical Co, Gillingham, Dorset, UK) or NB (Sigma) was added to SC+GC co-cultures or Sertoli cell only cultures 72 hours after the initial plating at a concentration of 10^{-4} M in dimethyl sulphoxide (DMSO, BDH Chemicals Ltd., Poole, Dorset, UK), at a final concentration of 0.3% (v/v), while controls received an equal volume of this vehicle. The same concentrations of toxicants were added to ST cultures at the start of the culture. Other ST were exposed to MAA (Aldrich Chemical Co) which was administered at a final concentration of 10^{-4} M. The concentrations of m-DNB, NB were chosen as being approximately equivalent to the peak levels, achieved *in-vivo* following administration of 50mg/kg m-DNB, 300mg/kg NB or 500mg/kg EGME, (see Allenby *et al.*, 1991b for discussion and references) which are known to cause severe disruption of spermatogenesis. Treatments caused no appreciable alteration of the culture medium pH. Where appropriate, 1µg/ml rat FSH-B2 or (NIADDK, USA) 250µU rFSH (Sigma) was added to either ST or SC+GC co-cultures respectively, at the time of toxicant or vehicle administration.

4.2.5. Measurement of newly synthesized proteins

Incorporation of 35 S-labelled methionine into secreted proteins (from either ST cultures or SC+GC co-cultures) was determined by precipitation of

aliquots of the culture medium with 10% (v/v) trichloroacetic acid, according to the methods described in Chapter 3.

4.2.6. Two-dimensional SDS PAGE

Newly synthesized ^{35}S -methionine labelled proteins were evaluated by 2-D SDS PAGE according to the methods described in Chapter 3.

4.2.7. Measurement of lactate secretion by SC+GC co-cultures

The concentration of lactate was measured in culture medium using the methods of Gutman and Wahlefeld (1974) and Czok and Lamprecht (1974), as modified by Williams and Foster (1988). These assays are based on the use of lactate dehydrogenase (LDH) and NAD or NADH, the end point being the change in absorbance measured at 340nm in a spectrophotometer.

Following the appropriate treatment of Sertoli cell+germ cell co-cultures over a 24 hour incubation, the culture medium was removed from each well. 2ml aliquots were deproteinized immediately with an equal volume of ice-cold 1M perchloric acid. The samples were then mixed and centrifuged at 2500g for 10 minutes at 4°C. The concentration of lactate was then measured in the decanted supernatant. For each experiment, standard solutions of lactate in the culture medium were prepared in an identical manner to the samples. The following solutions: 2ml 0.5M glycine/0.4M hydrazine buffer, pH 9.0, 0.2ml acidified sample or standard (25mM stock solution diluted with medium to give a range of standards = 0.2-5mM), 0.2ml 27mM NAD^+ solution and 0.02ml LDH (U/ml) solution, were added to a 4ml cuvette, mixed and incubated at 25°C for 1 hour. The increase in $A_{340\text{nm}}$ (the endpoint of the assay) was measured against a reagent blank, (with 0.2ml of perchloric acid replacing the sample), using a Perkin Elmer 5UV/vis spectrophotometer. The concentration of lactate in the samples was calculated by reference to a standard curve of $\Delta A_{340\text{nm}} \text{ v } [\text{lactate}] \text{ mM}$. Conditions of the assay are such that the LDH reaction is in favour of pyruvate and NADPH production i.e. hydrazine in the assay buffer. Hydrazine forms a complex with pyruvate which is not metabolized by LDH (Gutman and Wahlefeld, 1974).

4.2.8. SC+GC co-culture protein estimation

The concentration of protein was determined by the method of Bradford (1976). Following exposure of cultures to toxicants or FSH and the

subsequent removal of culture medium, 1ml sterile saline was added to each well and then sonicated. The content of each well was removed and stored at -20°C for protein estimation.

The amount of protein per well was measured using a commercially available kit (Protein Assay Kit, Bio-Rad Labs, UK) which uses bovine serum albumin (BSA, fraction C) as the standard. All protein samples per experiment were assayed simultaneously. This is a spectrophotometric assay, in which the absorbance of the solution is measured at a wavelength of 595nm against a saline blank in a PYE Unicam SP6-500 UV spectrophotometer. The concentration of protein was determined from the standard curve of A 595nm v [BSA] (mg/ml).

4.2.9. Measurement of cell exfoliation from SC+GC co-cultures

Using the criterion of toxicant-induced germ cell detachment described and validated previously by Gray and Beaman (1984) and Foster *et al.* (1987a), cell exfoliation was measured using a Coulter counter, (Coulter Electronics, Hemel Hempstead, UK) 24 hours after exposure of SG+GC co-cultures to the toxicant in question. Briefly, the Coulter counter withdraws 500µl of culture medium, diluted previously 1:10 (v/v) with isoton (TM) solution, through a photoelectric cell (window) and counts particulate material (mainly cells) passing through this window.

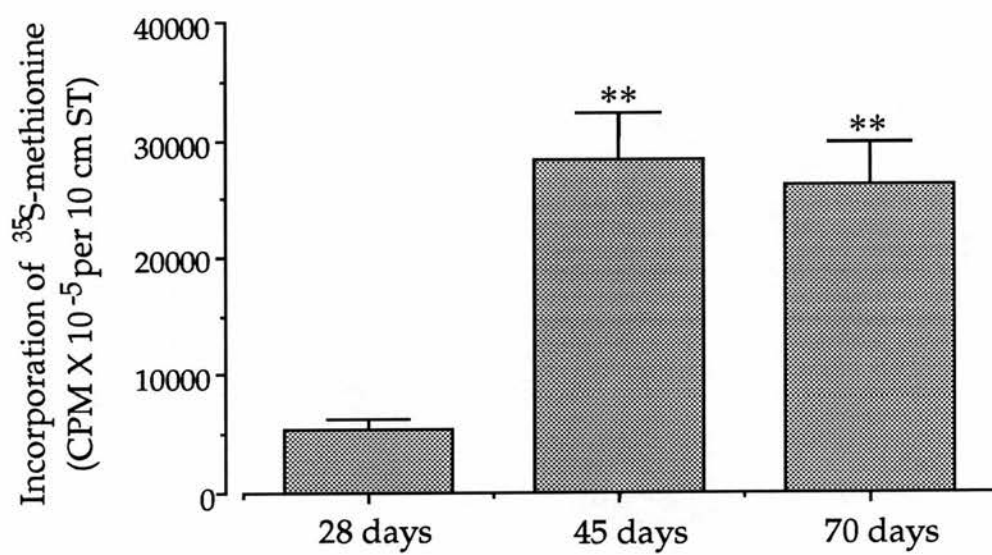
Exfoliated cells released from the Sertoli cell monolayer following treatment with toxicants were considered to be primarily germ cells, because an insignificant number of exfoliated cells was measured in the medium of Sertoli cell-only cultures following the addition of an identical dose of the same toxicant. The small number of cells exfoliated from the latter cultures is presumed to reflect detachment of the few residual germ cells that contaminate Sertoli cell cultures following Tris treatment (<10%) (Galdieri *et al.*, 1981).

4.3. Results

4.3.1. Overall incorporation of ³⁵S-methionine into secreted proteins

The overall level of incorporation of ³⁵S-methionine into secreted proteins by ST isolated from immature (aged 28 day), late pubertal (aged 45 days) and young adult (aged 70 day) rats is shown in Figure 4.1. The level of

Figure 4.1. Incorporation of ^{35}S -methionine over 24 hours into newly synthesized proteins secreted *in-vitro* by 10cm unstaged seminiferous tubules isolated from untreated rats aged 28, 45, 70 days and cultured for 24 hours. Values are means \pm SD of n=6. **<0.01, in comparison with 28 day group.



incorporation of radiolabel by immature rats was considerably less than that observed for late pubertal and young adult rats. SC+GC co-cultures showed a relatively low overall level of incorporation of ^{35}S -methionine into secreted proteins ($2116 \pm 770\text{cpm}/10^6$ Sertoli cells) but this could not be compared straight forwardly with the level of incorporation observed in isolated ST.

4.3.2. Effect of addition of FSH, or toxicants

The effect of the addition of FSH, m-DNB, NB or MAA to either ST isolated from rats aged 28, 45 or 70 days or SC+GC co-cultures obtained from rats aged 28 days is shown in Figures 4.2 and 4.3. The addition of FSH *in-vitro* had a small stimulatory effect on the overall incorporation of ^{35}S -methionine into secreted proteins by ST isolated from immature (aged 28 day) rats (Figure 4.2); addition of NB had no effect, whereas m-DNB and MAA caused a small but statistically insignificant increase in methionine incorporation (Figure 4.2). The same additions (FSH, m-DNB or NB) to SC+GC co-cultures resulted in a marked increase in incorporation of radiolabel in every instance (Figure 4.3), whereas the same additions to ST isolated from young adult (aged 70 day) rats had an inhibitory effect in every instance ($p < 0.01$ Figure 4.2). In late pubertal (aged 45 day) rats a picture similar to that seen in the adult was observed, although the toxicant-induced decreases in incorporation of ^{35}S -methionine were smaller than those observed in the adult and were only significant following treatment with FSH ($p < 0.05$ Figure 4.2).

4.3.3. Two-Dimensional SDS PAGE

Substantial differences were observed between the pattern of proteins secreted by ST and SC+GC from 28-day-old rats when compared with the pattern of proteins secreted by ST from adult rats aged 70 days. It is emphasized that all of these differences were confirmed in separate gels using medium from 3 separate experiments. Furthermore, the results obtained using 2-D SDS PAGE were in every instance highly repeatable. Many of the differences related to proteins of relatively low abundance but a number of the more prominent differences are circled and numbered in Figures 4.4 and 4.5 and listed in Table 4. Five proteins (numbered 1, 2, 4, 8 and 13) were detectable secretory products of ST from rats aged 28 days (protein 1 was not identifiable using SC+GC co-cultures) but their abundance increased considerably in the case of adult rats. A further two proteins (numbered 6, and 9), which were prominent secretory products of adult ST, were non-

Figure 4.2. Incorporation of ^{35}S -methionine over 24 hours into newly synthesized proteins secreted *in-vitro* by 10cm unstaged seminiferous tubules isolated from untreated rats aged 28, 45 or 70 days and cultured in the absence (control) or presence of either 10^{-4}M m-DNB, NB or MAA or $1\mu\text{g/ml}$ FSH. Incorporation has been expressed as a percentage of the mean control value (dashed line) obtained during the same experiment. Values are means \pm SD of $n=4$. * $p<0.05$, ** $p<0.01$, in comparison with respective control group.

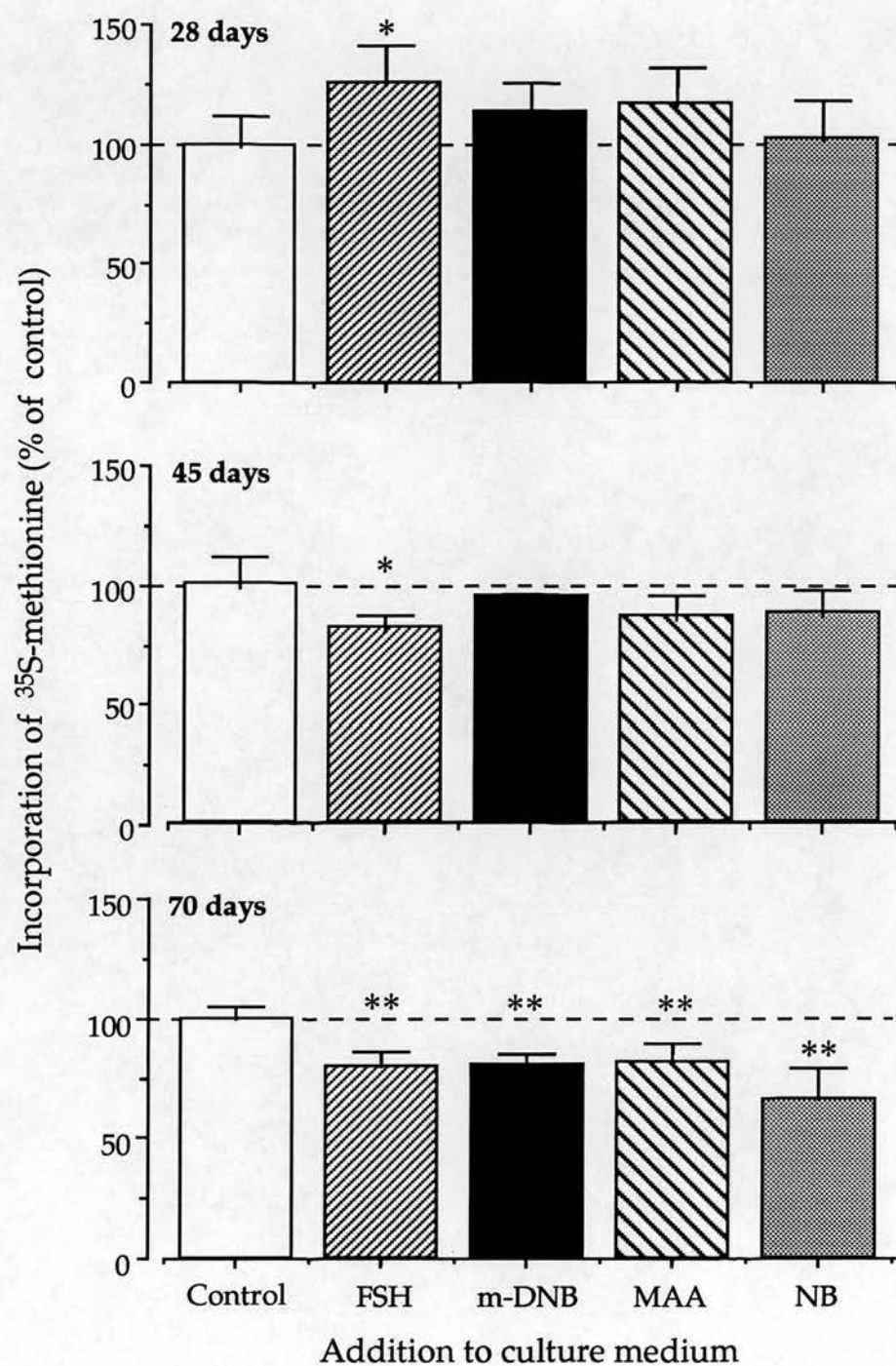
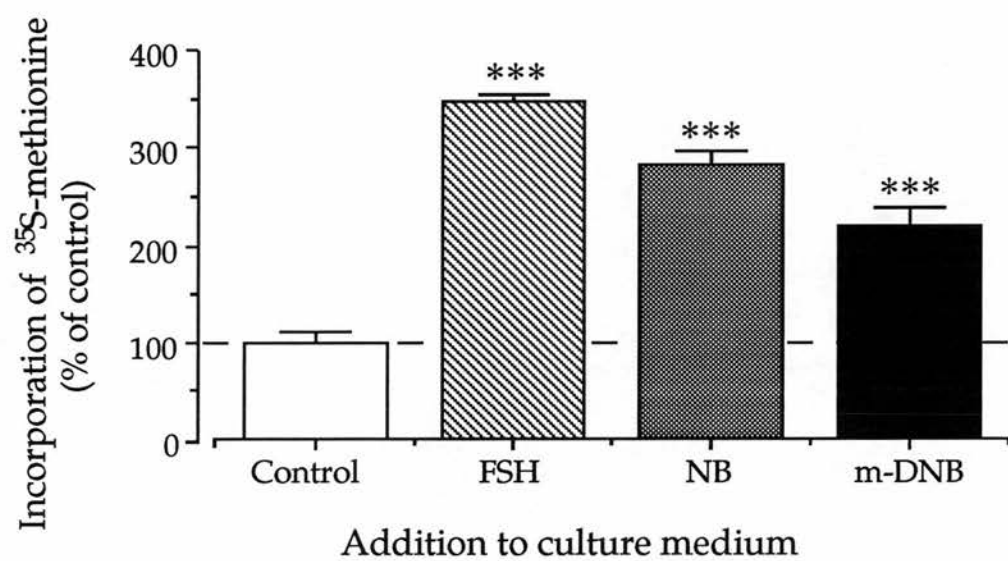


Figure 4.3. Incorporation of ^{35}S -methionine over 24 hours into newly synthesized proteins secreted *in-vitro* by Sertoli cell+germ cell co-cultures derived from rats aged 28 days which had been cultured in the absence (control) or presence of either 10^{-4}M m-DNB or NB or $250\mu\text{U}$ FSH. Incorporation has been expressed as a percentage of the mean control value (dashed line) obtained during the same experiment. Values are means \pm SD of $n=3$. *** $p<0.01$, in comparison with control.



Protein No	M _r (kDa)	pI	Effect of m-DNB or NB	Age-dependence	Possible Identity
1	63 - 70	5.6 - 5.9	Decrease	Increase with Age	unknown
2	38 - 38.5	5.8 - 5.9	—	Increase with Age	ARP-4 (Sharpe <i>et al.</i> , 1992)
3	28 - 30	5.8 - 6.9	Increase	Decrease with Age	Possible charge isomers of SGP-2
4	21- 24	5.4 - 5.9	Decrease	Increase with Age	unknown
5	20 - 27	5.8 - 6.3	Increase	Absent in Adult	unknown
6	22 - 30	6.9 - 7.4	Decrease	Present only in adult	ARP-3 (Sharpe <i>et al.</i> , 1992)
7	17.5 - 20	5.3 - 5.9	Decrease	Present only in adult	unknown
8	17- 20	5.9 - 6.4	Decrease	Increase with Age	unknown
9	13 - 14.5	6.9 - 7.6	Decrease	Present only in adult	ARP-2 (Sharpe <i>et al.</i> , 1992)
10	12.5 - 15	6.3 - 6.5	Decrease	Decrease with Age	unknown
11	12.5 - 15	6.4 - 6.9	Decrease	Decrease with Age	unknown
12	12.5 - 15	6.9 - 7.0	Decrease	Decrease with Age	unknown
13	~9.5	6.9 - 7.4	Decrease	Increase with Age	ARP-1 (Sharpe <i>et al.</i> , 1992)

Table 4. List of seminiferous tubule-secreted proteins which showed major age-dependent differences in relative abundance and the effect of exposure to the Sertoli cell toxicants m-DNB and NB. Protein numbers correspond to those shown in Figures 4.4. 4.5. 4.6. and 4.7.

Figure 4.4. Comparison using 2-dimensional SDS PAGE of proteins secreted by Sertoli cells+germ cells (SC+GC) isolated from rats aged 28 days (middle) or unstaged ST isolated from rats aged 28 (top) or 70 (bottom) days. Proteins which showed major repeatable age-dependent differences are circled and numbered on each of the gels. The positions of the three major Sertoli cell-secreted proteins, sulphated glycoprotein-1 (SGP-1), sulphated glycoprotein-2 (SGP-2) and cyclic protein-2 (CP-2), are indicated for reference.

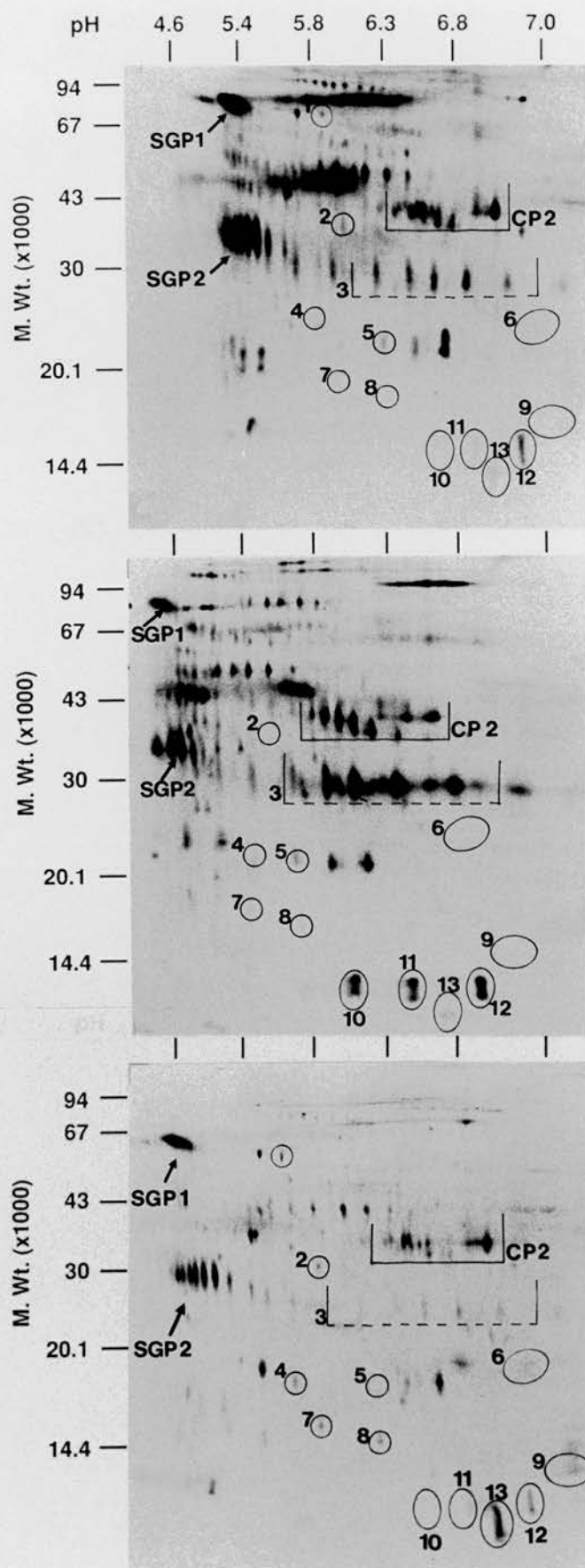
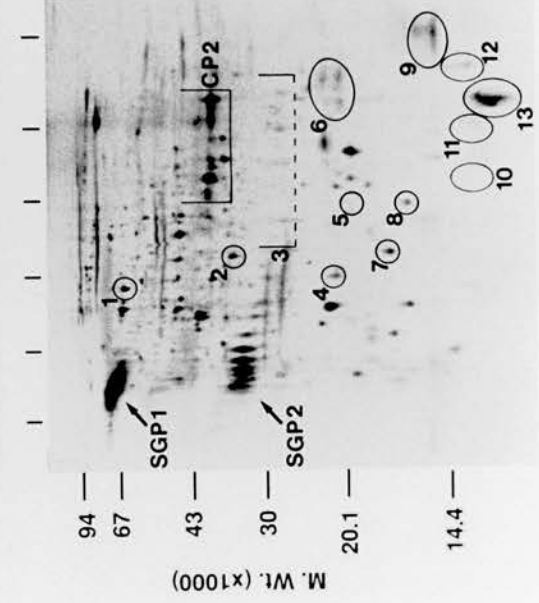
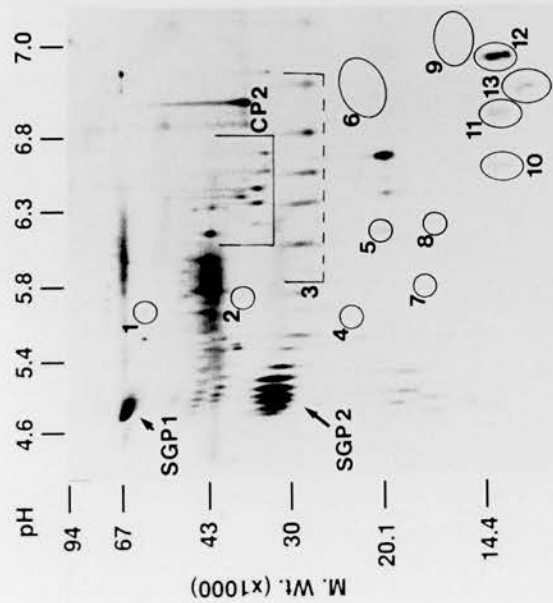
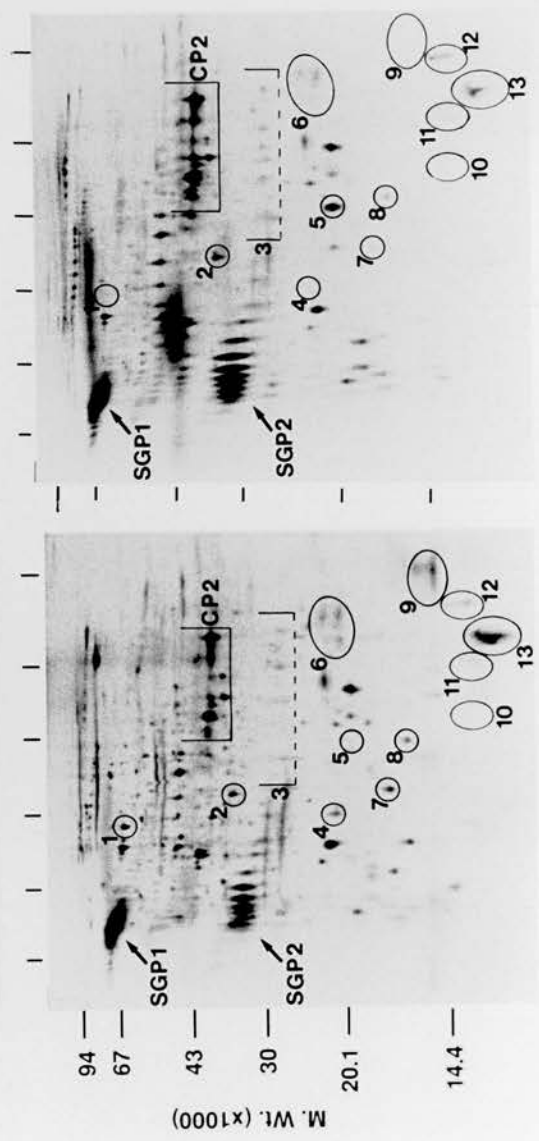
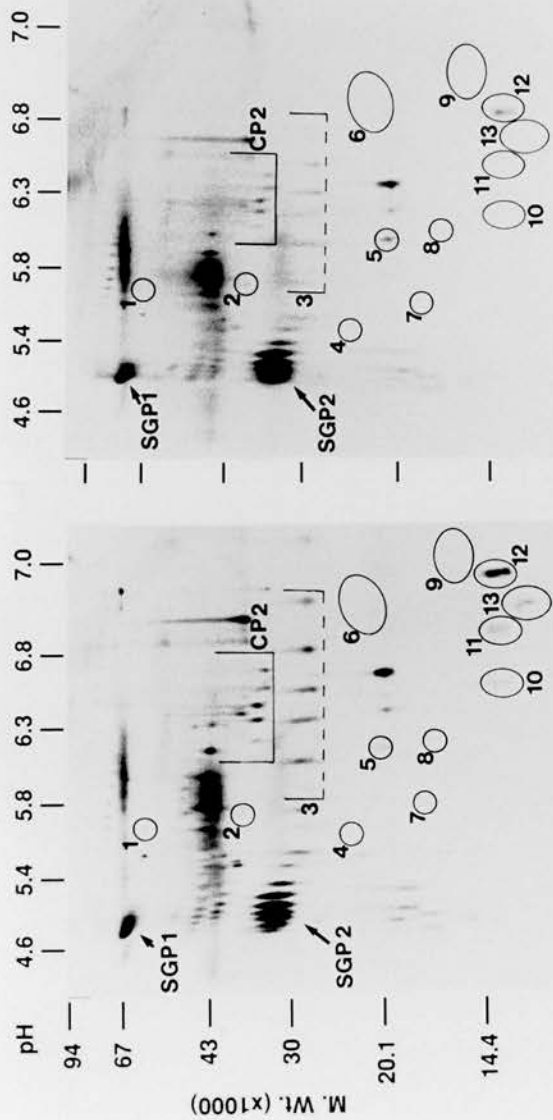


Figure 4.5. Comparison using 2-dimensional SDS PAGE of the effect of m-DNB (10^{-4}M) on proteins secreted by unstaged ST isolated from immature (28 days - top) or adult (70 days - bottom) rats and cultured in the absence (left) or presence (right) of m-DNB. Proteins which showed major changes following m-DNB exposure are circled and numbered. The positions of the three major Sertoli cell secreted proteins, sulphated glycoprotein-1 (SGP-1), sulphated glycoprotein-2 (SGP-2) and cyclic protein-2 (CP-2), are indicated for reference.



detectable using ST or SC+GC from immature rat. It is of interest that the secretion of four of these proteins (numbered 2, 6, 9 and 13) has been shown to be androgen-regulated in the adult (see Table 1). It is also emphasized that the substantial age-dependent differences in relative abundance of these proteins, as shown on the fluorograms (which compared equal amounts of radiolabelled protein), underestimate by a factor of five the real magnitude of difference in total secretion of these proteins, as the overall incorporation of ^{35}S -methionine into ST-secreted proteins was 5 fold greater for adult than for immature rats (Figure 4.1).

Other proteins showed an age-dependent decline in secretion between 28 and 70 days of age (numbered 3, 5, 10, 11 and 12 in Figures 4.4 and 4.5). Of these proteins, 3 and 5 were of particular interest. The former of these existed as a prominent band of charge isomers of approximately 28-30 kilodaltons and may represent high pI forms of sulphated glycoprotein-2 (SGP-2). This band of proteins was a particularly prominent feature of SC+GC co-cultures from immature rats but was a minor component of the proteins secreted by ST from adult rats. The pattern of proteins secreted by SC+GC cultures from immature rats was generally comparable to that for isolated ST from rats of the same age, especially in their comparison with the adult. However, a number of proteins were more prominent secretory products of the SC+GC co-cultures than of isolated ST, and these included proteins 3, 10, 11 and 12 (Figure 4.4). This difference may be related to the presence of relatively fewer germ cells in the SC+GC co-cultures than in the ST (see Discussion). Protein 5, which had a molecular weight of approximately 26 kilodaltons, was non-detectable as a secretory product of normal adult ST but could be induced by culture of the ST for 24 hours with either m-DNB or NB (Figures 4.5 and 4.6), but not with MAA or FSH (Figure 4.7). Indeed, exposure of ST from adult rats to either m-DNB or NB also caused disappearance of, or substantial decreases in the secretion of all of the proteins described except for numbers 2, 3 and 5, but had relatively little effect on proteins secreted by ST from immature rats, in which most of these proteins were absent or of low abundance even in the control situation (Figure 4.5). Addition of MAA or FSH to adult ST cultures resulted in a slight decrease in the secretion of proteins numbered 4, 7, 8 and 9 and 4, 7, 8 and 13 respectively (Figure 4.7) but had very relatively little effect on proteins secreted by ST from immature rats, with the exception of protein number 1 which was increased slightly (Figure 4.8).

Figure 4.6. Comparison using 2-dimensional SDS PAGE of the effect of NB (10^{-4}M) on proteins secreted by unstaged ST isolated from adult (70 days) rats and cultured in the absence (top) or presence of NB (bottom). Age-dependent proteins which showed major changes following NB exposure are circled and numbered. The positions of the three major Sertoli cell-secreted proteins, sulphated glycoprotein-1 (SGP-1), sulphated glycoprotein-2 (SGP-2) and cyclic protein-2 (CP-2), are indicated for reference.

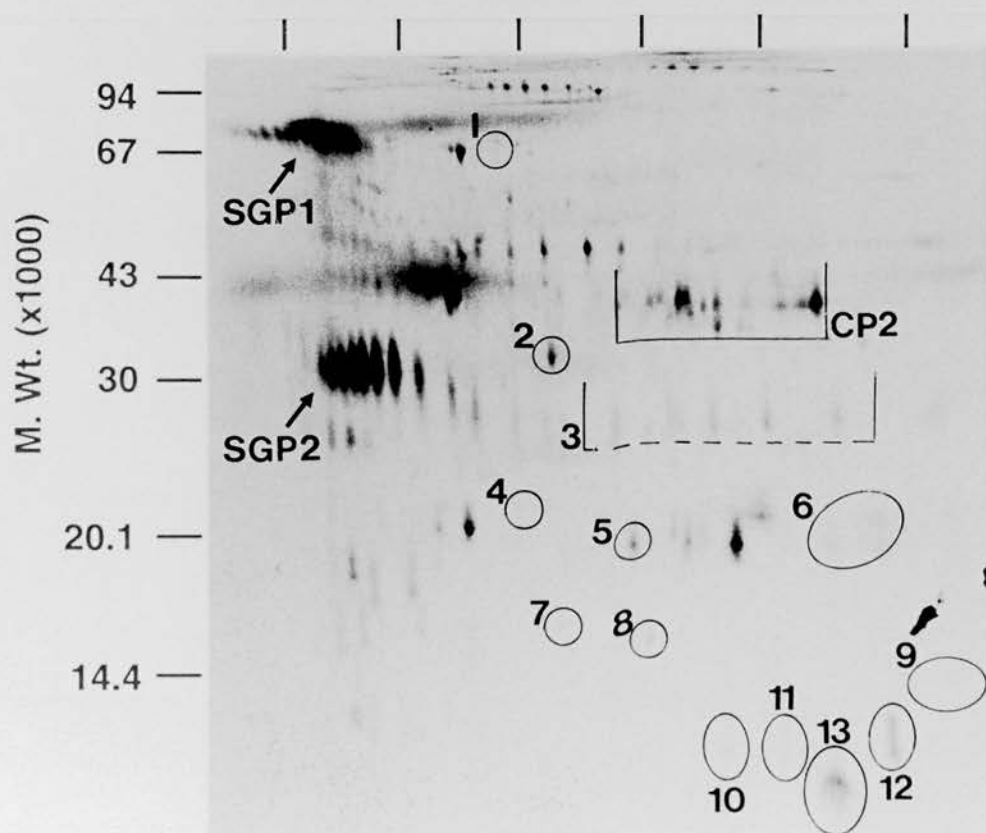
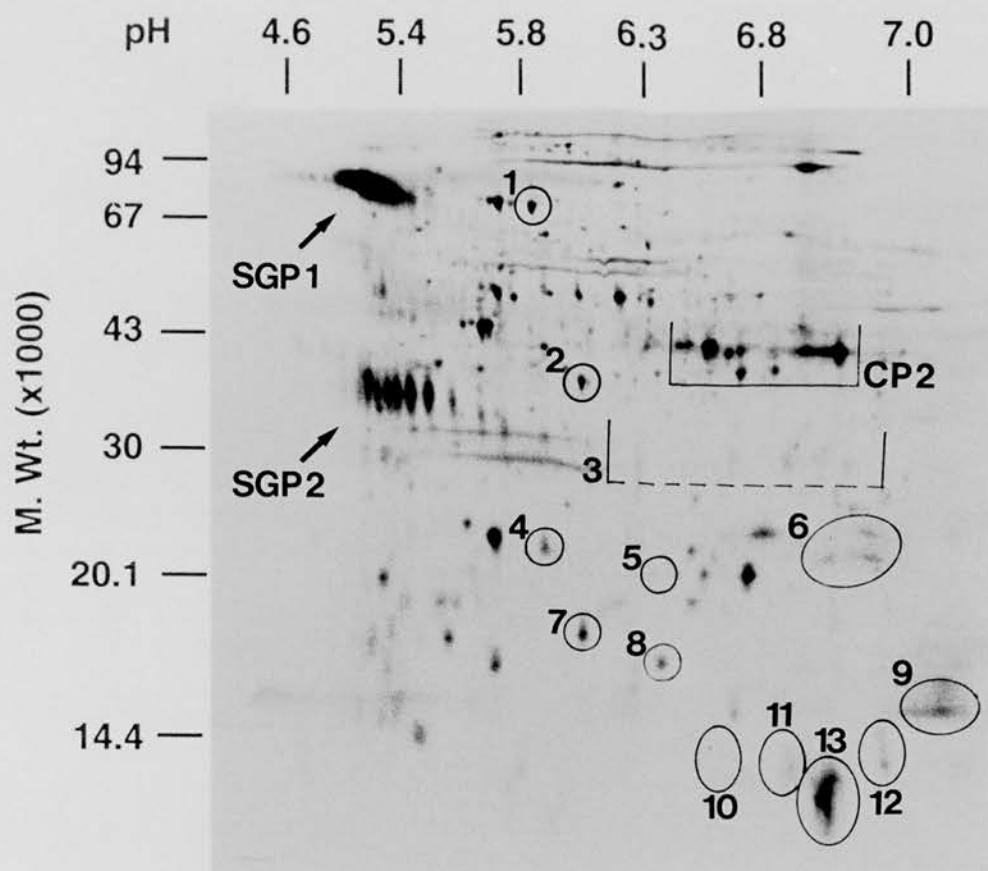


Figure 4.7. Comparison using 2-dimensional SDS PAGE of the effect of MAA (10^{-4}M) or FSH ($1\mu\text{g/ml}$) on protein secretion by unstaged ST isolated from immature (28 day) rats and cultured in the absence (top) of or in the presence of MAA (middle) or FSH (bottom). Proteins which showed major repeatable age-dependent differences (see Figure 4.4) are circled and numbered on each of the gels. The positions of the three major Sertoli cell secreted proteins, sulphated glycoprotein-1 (SGP-1), sulphated glycoprotein-2 (SGP-2) and cyclic protein-2 (CP-2), are indicated for reference.

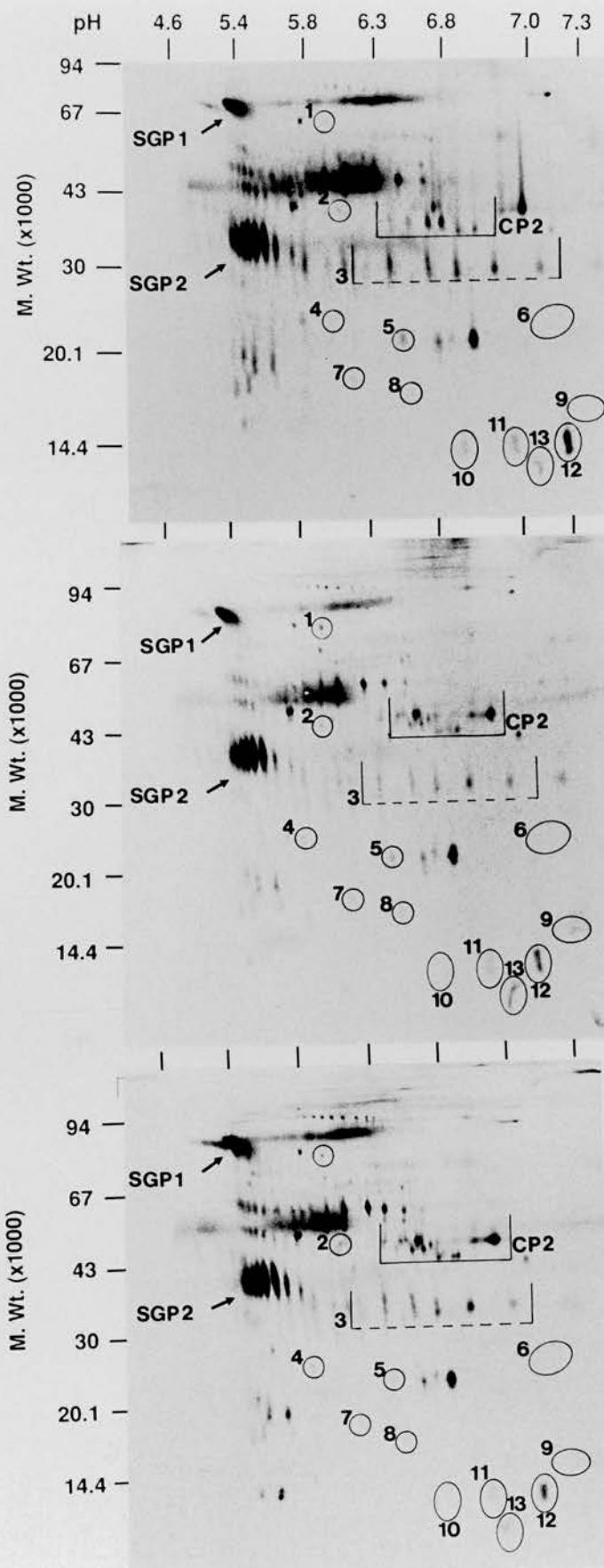


Figure 4.8. Comparison using 2-dimensional SDS PAGE of the effect of MAA (10^{-4}M) or FSH ($1\mu\text{g/ml}$) on protein secretion by unstaged ST isolated from mature (70 day) rats and cultured in the absence (top) of or in the presence of MAA (middle) or FSH (bottom). Proteins which showed major repeatable age-dependent differences (see Figure 4.4) are circled and numbered on each of the gels. The positions of the three major Sertoli cell secreted proteins, sulphated glycoprotein-1 (SGP-1), sulphated glycoprotein-2 (SGP-2) and cyclic protein-2 (CP-2), are indicated for reference.

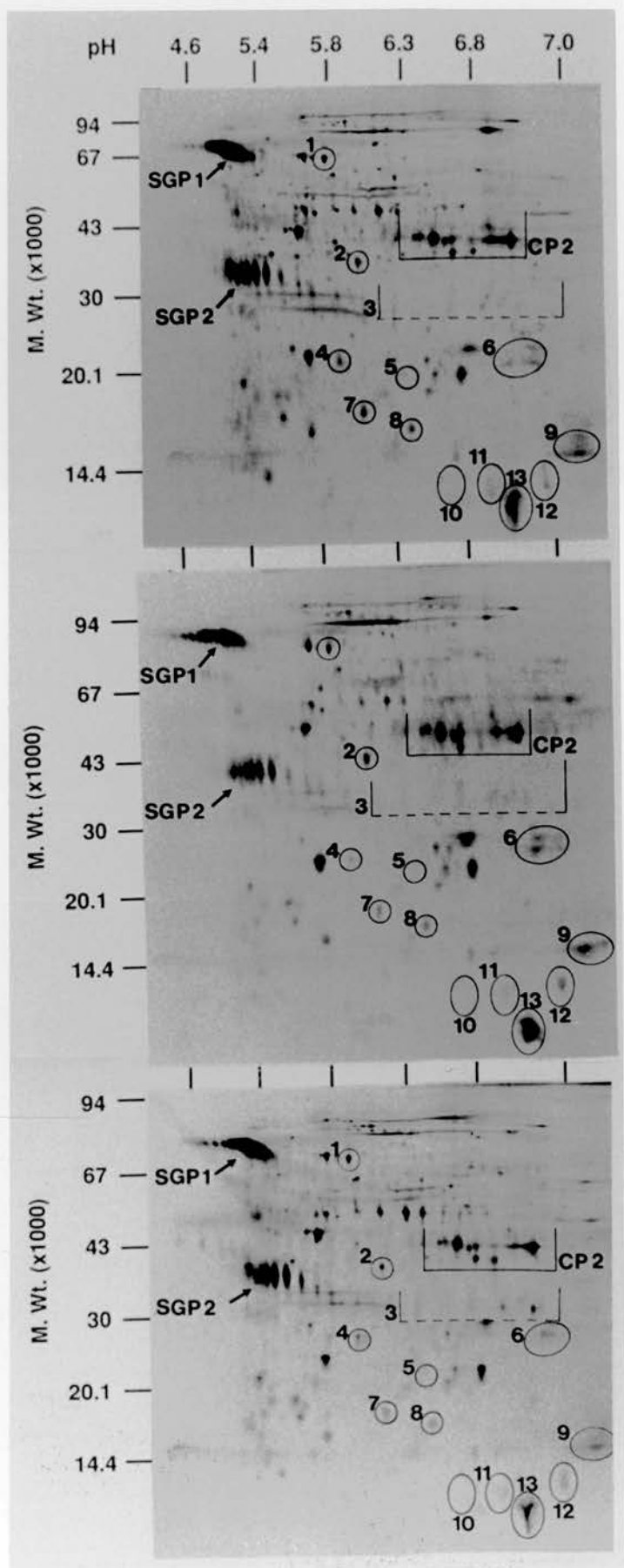


Figure 4.9 Effect of m-DNB or NB (both 10^{-4}M) or vehicle alone (DMSO - 0.30% v/v) on the number of detached cells present in the culture medium of Sertoli cell+germ cell co-cultures (top) or Sertoli cell only cultures (bottom), following a 24 hour exposure period. Values are means \pm SD for 6 replicates. *** $p < 0.001$.

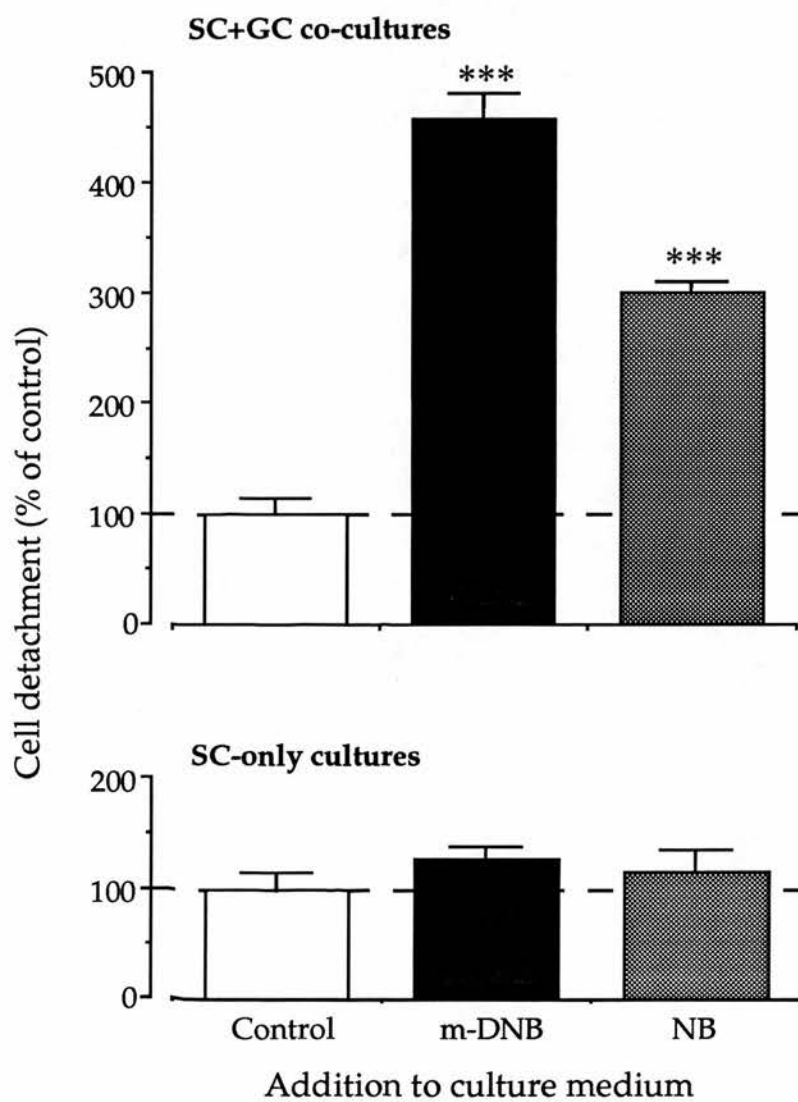
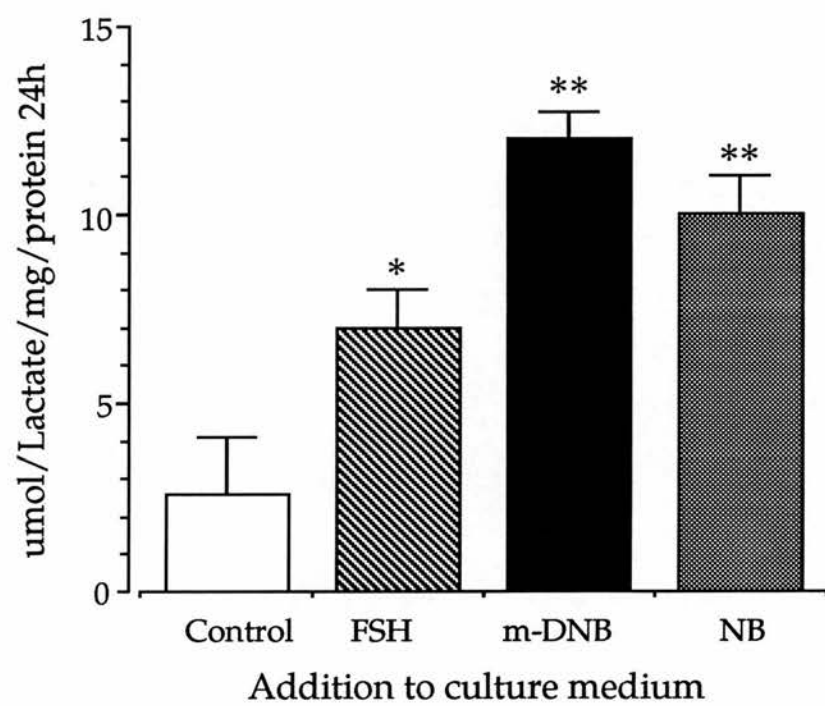


Figure 4.10. Effect of m-DNB or NB (both 10^{-4}M) or FSH ($250\mu\text{U}$) on the secretion of lactate into the medium of Sertoli cell+germ cell co-cultures following a 24 hour exposure period. Control cultures received vehicle alone (DMSO - 0.30% v/v). Values are means \pm SD for 6 replicates. * $p<0.05$, ** $p<0.01$ in comparison with control.



4.3.4. Effect of addition of m-DNB or NB on cell exfoliation

Cell exfoliation following addition of m-DNB or NB to Sertoli cell only and SC+GC co-cultures is shown in Figure 4.9. The addition of m-DNB or NB (both at 10^{-4} M) to SC+GC co-cultures for 24 hours resulted in significant increases in the exfoliation of germ cells into the culture medium ($p < 0.001$). These exfoliated germ cells were considered to be primarily germ cells, as an insignificant number of exfoliated cells was measured after the exposure of Sertoli cell monolayers to an identical dose of m-DNB or NB (Figure 4.9).

4.3.5. Effect of m-DNB, NB or FSH on secretion of lactate

The addition of m-DNB and NB (both at 10^{-4} M) to SC+GC co-cultures for 24 hours resulted in significant increases ($p < 0.01$) in the secretion of lactate into the culture medium (Figure 4.10). A similar, though smaller, increase was observed following treatment with FSH ($p < 0.05$).

4.4. Discussion

The objectives of the experiments described in this chapter were to identify changes in the pattern of protein secretion by seminiferous tubules (ST) isolated from rats of different ages and to evaluate the effects of agents known either to support (FSH) or to impair (testicular toxicants) the process of spermatogenesis.

Sertoli cells are known to play a key role in the complex process of spermatogenesis by providing a unique environment essential for germ cell growth and differentiation (Dym and Fawcett, 1970). It is recognized also that Sertoli cells undergo a number of morphological and functional changes, during testicular maturation (Steinberger *et al.*, 1978; Sanborn *et al.*, 1986), which coincide with the appearance of maturing germ cells and the onset of meiosis (Steinberger *et al.*, 1978, Salhanick and Weibe, 1980), and this suggests that germ cells are partly responsible for these changes. It has been shown, for instance, that the addition of enriched preparations of particular germ cell types (pachytene spermatocytes or round spermatids) to immature Sertoli cell cultures modulates the secretion of a number of Sertoli cell products. Le Magueresse *et al.* (1986) showed that both pachytene spermatocytes and round spermatids were capable of enhancing basal and FSH-stimulated ABP

secretion when they were co-cultured directly with Sertoli cells. Moreover, Le Magueresse and Jégou (1988) found that addition of germ cell conditioned medium to immature rat Sertoli cell cultures stimulated ABP secretion and inhibited oestradiol production. Similarly, transferrin secretion and transferrin mRNA levels in Sertoli cells are stimulated by either co-culture with germ cells or germ cell-conditioned media (Le Magueresse *et al.*, 1988; Stallard and Griswold, 1990).

These observations suggest that germ cells are able to influence the secretory function of the Sertoli cell. However, the majority of studies have used Sertoli cells which have been isolated from immature animals and there is now increasing evidence to suggest that the effect of germ cells on the secretory activity of the Sertoli cell changes with increasing age (Castellón *et al.*, 1989a,b). It is not certain that an extrapolation from immature to adult animals can be made. For instance, studies with Sertoli cells isolated from immature rats and stimulated with dibutyryl cyclic AMP have shown that the addition of round spermatids (and, to a small extent, pachytene spermatocytes) or round spermatid-conditioned medium is able to stimulate both the secretion of immunoactive and bioactive inhibin and the level of mRNA for the α -subunit of inhibin (Pineau *et al.*, 1990). This is in marked contrast to the results obtained by Allenby *et al.* (1991a), who showed that the secretion of immunoactive inhibin by adult rat Sertoli cells (in seminiferous tubules) was regulated by elongate spermatids (and possibly pachytene spermatocytes). Taken together, results such as these imply that the response of the Sertoli cell to germ cells is dependent on the stage of maturation of the Sertoli cell, which is probably determined by the type of germ cells present. In the study by Pineau *et al.* (1990), Sertoli cells were isolated from immature 20-day-old rats. At this age pachytene spermatocytes would have been the most mature germ cells to which the Sertoli cell would have been exposed *in-vivo*. Exposure of these cells *in-vitro* to more mature germ cell types (round spermatids) exerted a stimulatory effect on inhibin secretion whereas, in the adult testis, this role is taken over by the most mature germ cells present (the elongate spermatids). In support of this hypothesis it has been shown that the stimulatory effect of added germ cells on the secretion of ABP by Sertoli cells (obtained from rats aged 10, 15, 20 and 45 days) increased noticeably with age and, in particular, that round spermatids only exerted significant effects at the two greatest ages (Le Magueresse and Jégou, 1988). Based on observations such as these it has been concluded that the modulation of

Sertoli cell function during development may be transferred sequentially to the most mature germ cell type present within the epithelium (Jégou, 1991).

To date, most studies have used isolated Sertoli cells or Sertoli cells which have been co-cultured with a particular class of germ cell. This is not the most satisfactory approach, especially as it now appears that the presence of other germ cell types (and other cell types) may co-ordinate the secretory functions of the Sertoli cell. In the present study, isolated ST were used because they offer two distinct advantages over the use of isolated cells. Firstly, they can be isolated with an intact peritubular cell layer so as to maintain, as far as is possible, the cytoarchitecture and cell-cell interactions which would be found normally *in-vivo* (Sharpe *et al.*, 1990; Allenby *et al.*, 1991a,b) and, secondly, ST can be isolated from both mature and immature rats relatively easily, whereas Sertoli cells can only be isolated with relative ease from immature rats. In this study a pool of unstaged ST was isolated from rats in the three age groups, as it is impossible to distinguish the various stages of the spermatogenic cycle at 28 days of age using the normal criteria (Parvinen, 1982). The present study has also evaluated the susceptibility of ST from rats of different ages to the effects of three well-characterized testicular toxicants, meta-dinitrobenzene (m-DNB) (Foster *et al.*, 1986; Blackburn *et al.*, 1988; Hess *et al.*, 1988; Allenby, 1990) and nitrobenzene (NB) (Bond *et al.*, 1981; Levin *et al.*, 1988; Allenby *et al.*, 1990), both of which have been shown to exert their adverse effects on spermatogenesis via perturbation of the Sertoli cells, and methoxyacetic acid (MAA), a germ cell specific toxicant, which causes selective and stage-specific degeneration of pachytene spermatocytes (Foster *et al.*, 1983; Creasy *et al.*, 1985).

The addition of FSH, m-DNB, NB or MAA to ST isolated from rats aged 28 days either had no effect (NB) or had a small stimulatory effect (FSH, m-DNB, and MAA) on the overall incorporation of ³⁵S-methionine into secreted proteins. The same additions to SC+GC co-cultures resulted in significant increases in incorporation of radiolabel in every instance. This was in marked contrast to the results obtained following the isolation of ST from adult rats, in which the same additions had an inhibitory effect in every instance (Figure 4.2). ST from pubertal rats showed a similar trend to those of the adult (i.e. all additions decreased methionine incorporation) but, overall, their response was probably intermediate, between that observed for ST from rats aged 28 and that observed for ST from rats aged 70 days. Due to the different isolation and culture procedures, it is possible that isolated ST and isolated Sertoli cells (with or without isolated germ cells) may respond

differently *in-vitro*. SC+GC co-cultures showed greater stimulation of ^{35}S -methionine into secreted proteins following exposure to FSH, m-DNB or NB than isolated ST from rats of the same age. This could be due to differences in the endogenous pool size of radioinert methionine between ST and SC+GC, for example. However, the stimulatory effect on protein secretion observed in immature rats, following exposure to FSH and testicular toxicants, is in general agreement with the results of previous studies, obtained using either Sertoli cell endpoints such as the secretion of lactate and pyruvate (Chapin *et al.*, 1986; Williams and Foster, 1988), inhibin (Allenby *et al.*, 1990; 1991b) and androgen-binding protein (Galdieri *et al.*, 1984; Le Magueresse *et al.*, 1986; Le Magueresse and Jégou, 1988) by cultures of isolated Sertoli cells or Sertoli cells+germ cells (SC+GC) from immature rats. In the present study the secretion of lactate was used as a positive control. Exposure of SC+GC co-cultures to FSH, m-DNB or NB at a single dose level increased the secretion of lactate to a similar extent to that reported by previous investigators (Williams and Foster, 1988; Allenby, 1990). The stimulatory effects shown in this and previous studies are unlikely to be artifacts of the *in-vitro* culture system as similar stimulatory effects on the secretion of inhibin have also been shown *in-vivo* following toxicant exposure (Allenby *et al.*, 1991a). The reason for the age-dependent differences in the response of overall protein secretion to toxicant exposure is unknown but, as the morphological and biochemical characteristics of the Sertoli cells are known to change with age (Castellón *et al.*, 1989a,b; Jégou, 1991), it is thought to reflect a fundamental, maturational difference which appears during puberty (and is evident in late pubertal rats aged 45 days) and which is probably related to the appearance of the more mature germ cell types. Similarly, the responsiveness of the Sertoli cell to FSH stimulation has been shown to be age-dependent (Means *et al.*, 1976; Steinberger *et al.*, 1978; Le Magueresse and Jégou, 1988). The fact that the exposure of ST from immature and adult rats to FSH showed similar differences to those observed for the testicular toxicants further supports the view that differences in protein secretion between immature and adult rats, which this study has shown are truly age-dependent differences, are not the result of differences in the susceptibility to the adverse effects of testicular toxicants between Sertoli cells and isolated ST.

Analysis of ST-secreted proteins by 2-dimensional SDS PAGE revealed substantial differences with age in the proteins secreted by isolated ST and their relative abundance. Although age-dependent differences in protein secretion have been studied previously (Zabludoff *et al.*, 1990), the focus of

their work was on CP-2. CP-2, SGP-1 and SGP-2 (all Sertoli cell-secreted proteins; see Griswold, 1988) were prominent secretory products in both immature and adult rats in the present study. However, many of the moderately abundant proteins secreted by ST from adult rats were non-detectable or only minor products of ST from immature rats. The secretion of four of these proteins (numbered 2, 6, 9 and 13 in Figure 4.4) is known to be acutely androgen-dependent (Sharpe *et al.*, 1992) and germ cell dependent (McKinnell and Sharpe, 1992) in the adult rat; these proteins were not particularly prominent in the present study because unstaged ST were used. It is thought that most of the ST-secreted proteins which have been identified as differing between adult and immature rats are germ cell-dependent i.e. their secretion decreases (or increases) in the adult when a particular germ cell type is depleted (Sharpe and McKinnell, unpublished data). This may mean either that the proteins in question emanate from one or more types of germ cells (which have degenerated) or that their secretion by the Sertoli cell is germ cell-dependent. Probable examples of the latter are proteins 3 and 5, the secretion of which increased in the relative absence of germ cells, being most prominent in SC+GC co-cultures from immature rats, still prominent but less so in ST from immature rats and absent or of very low abundance in the adult (Figure 4.4). Removal of two generations of germ cells from the ST of adult rats leads to a substantial increase in the secretion of protein 3 (McKinnell and Sharpe, unpublished data). Indeed, increased secretion of proteins 3 and 5 can be induced by exposure to Sertoli cell toxicants such as m-DNB and NB either *in-vitro* (Figures 4.5 and 4.6) or *in-vivo* (see Chapters 5 and 6, respectively). The identity of proteins 3 and 5 is unknown, although it is possible that protein 3 represents isomers of SGP-2 with a high pI. If this is the case then it suggests that the degree of sulphation and/or glycosylation of SGP-2 (which probably determines its pI and M_r) is germ cell-regulated.

The present data have shown that exposure of adult ST *in-vitro* to two known Sertoli cell toxicants, m-DNB and NB, induced a marked decrease in the secretion of a number of specific proteins (numbers 1, 4, 6, 7, 8, 9 and 13), most of which were undetectable, or were only minor, components of ST-secreted proteins in immature rats. However, other proteins (numbered 10, 11 and 12), which were more prominent secretory products of ST from immature than of ST from adult rats, also decreased in relative abundance following toxicant exposure *in-vitro* (Figure 4.5). In contrast, protein 5 increased in abundance following culture of ST from immature rats with m-DNB and a similar change was noted for the adult, although this protein was

not detectable in adult controls. Exposure of adult rat ST *in-vitro* to MAA or FSH resulted in a slight decrease in the secretion of proteins numbered 4, 7, 8 and 9 and 4, 7, 8 and 13 respectively (Figure 4.7). The cellular source of proteins numbered 1, 4, 6, 7, 8, 9 and 13 remains to be established. From the present studies it is not possible to say whether these proteins derive from Sertoli or germ cells. However, a recent study by McKinnell and Sharpe (1992) has shown that the secretion of protein number 6 is decreased when pachytene spermatocytes are depleted selectively from the seminiferous tubule, whilst proteins numbered 4 and 9 are absent or decreased markedly when round spermatids are missing from the tubule. On the basis of these results it may be suggested that protein 6 and proteins 4 and 9 are secretory products of pachytene spermatocytes and round spermatids, respectively, or that they are products of Sertoli cells, the secretion of which is absolutely dependent upon the presence of pachytene spermatocytes or round spermatids. It is likely that some of the toxicant-induced changes in ST protein secretion are responsible for the subsequent adverse changes in spermatogenesis which they induce *in-vivo*.

In conclusion, the experiments presented in this chapter have shown that both the pattern and the total level of protein secretion is very different between immature and adult rats, as is their response to FSH (Means *et al.*, 1986; Steinberger *et al.*, 1978; Le Magueresse and Jégou, 1988) and toxicants. It is likely that these differences are a direct consequence of changes in the complement of germ cells with age, and this adds to the growing body of evidence supporting the argument that germ cells play a central role in modulating the secretory function of the Sertoli cells (Jégou *et al.*, 1992; Sharpe, 1993). As the major objective of this thesis was to develop markers of early damage to spermatogenesis in the adult, it was considered appropriate to use only ST from mature animals.

5. Effect of m-DNB on ST protein secretion

The previous chapter has demonstrated changes in the secretion of proteins by unstaged ST following toxicant exposure *in-vitro*. As it is known that Sertoli cell secretory function changes according to the stage of the spermatogenic cycle (Parvinen, 1982, 1993), it is presumed that some of these functions are either altered selectively or are more sensitive to the adverse effects of particular chemicals. This chapter describes studies which assessed this possibility by identifying whether exposure to the Sertoli cell toxicant meta-dinitrobenzene caused stage-specific changes in the secretion of proteins by isolated ST.

5.1. Introduction

Dinitrobenzenes (DNB) are aromatic nitro compounds that are used primarily as chemical intermediates in the synthesis and manufacture of a large number of commercially important organic compounds, certain dyes and dye intermediates. In addition, dinitrobenzene is used in the manufacture of explosives and, to a limited extent, in the plastics industry. Dinitrobenzene is obtained by the nitration of nitrobenzene, which results in a mixture of the ortho, meta and para isomers of the nitro group ring substitutions. Of the three isomers meta dinitrobenzene (m-DNB) is the most important economically. In common with many other nitroaromatic compounds, exposure to the meta and para isomers of DNB can induce methaemoglobinaemia, with resulting cyanosis in both experimental animals (Facchini and Griffiths, 1981; Blackburn *et al.*, 1988) and man (Beritic, 1956; Ishihara *et al.*, 1976) and, on prolonged exposure, can result in anaemia (Watanabe *et al.*, 1976).

m-DNB is a lipid soluble and weakly acidic compound and has been shown to be the most toxic of the three isomers of DNB to humans; its toxicity has been known for some 122 years (Starkow, 1871). m-DNB is readily absorbed through intact skin (Ishihara *et al.*, 1976) and is also absorbed rapidly through the lungs and thence into the blood stream. The lethal dose in humans is probably in the range 5-50mg/kg (Gosselin *et al.*, 1984) and, in rats, the oral LD₅₀ is 83mg/kg (Cody *et al.*, 1981).

Cody *et al.* (1981) reported that, when m-DNB is administered at 20-200mg/l (approximately 2.6-13mg/kg/day) in drinking water for 8-16 weeks it decreased testicular weight significantly and affected spermatogenesis adversely in addition to the expected haematological effects.

Using this route of exposure it is, however, virtually impossible to evaluate accurately the dose each animal received or to equate the dose ingested with the degree of testicular disruption. Furthermore, these studies failed to establish, 1) the minimum effective dose and time response which would result in testicular disruption, 2) the initial testicular lesion, 3) whether it is the parent compound or a metabolite, or both, which is or are responsible for the toxicity, or 4) the mode of action of m-DNB.

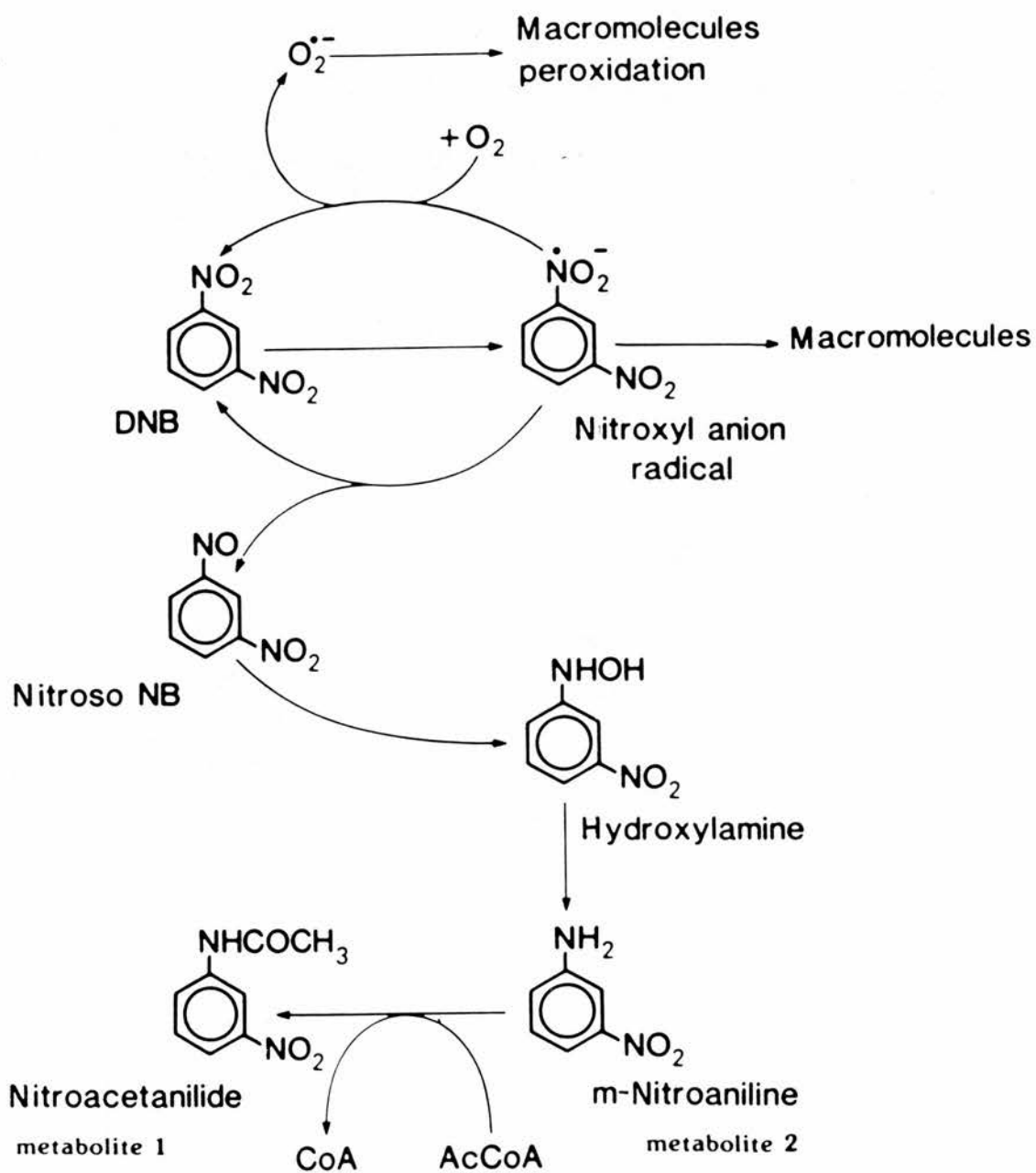
Preliminary investigations by Blackburn *et al.* (1985) indicated that m-DNB produced both time- and dose-dependent decreases in testicular weight within 24 hours of a single oral dose of 15 or 25mg/kg. Histological evaluation indicated disruption of the seminiferous epithelium, with the initial lesion occurring within the Sertoli cell accompanied by germ cell degeneration. A more detailed study, using electron microscopy (Foster *et al.*, 1986) of the effects of a single oral dose of m-DNB to rats, revealed vacuolation of Sertoli cell cytoplasm particularly at stages IX and X of the spermatogenic cycle within 24 hours of dosing. This occurred in the absence of morphological changes to germ cells. Studies using m-DNB *in-vitro* in testicular cell cultures showed a similar morphological response to that observed *in-vivo* at concentrations which were equivalent to or lower than the peak levels of radioactivity observed in blood (50 μ M) and testis (20 μ M) after a single oral dose of m-[¹⁴C]-DNB. This was characterized by Sertoli cell vacuolation coupled with the exfoliation of viable germ cells, together with the presence of 'inclusion bodies' of degenerate germ cells in vacuoles contained within the Sertoli cell. These changes were directly comparable with the changes observed *in-vivo* after administration of m-DNB. The failure of the Sertoli cell monolayer to sustain testicular germ cells (reflected in the detachment of viable germ cells) was identical to the *in-vitro* effect of other compounds for which the initial lesion *in-vivo* has been shown to be the Sertoli cell. Such compounds include certain phthalate esters (Foster *et al.*, 1982) and AF 1312/TS (an imidazole compound; De Martino *et al.*, 1975). Having established a morphological response by the addition of m-DNB *in-vivo*, further metabolic studies were conducted with radiolabelled compound *in-vitro*. In these studies, varying concentrations of ¹⁴C-labelled m-DNB were added to Sertoli cell+germ cell co-cultures (Lloyd and Foster, 1987). Aliquots of culture media were analysed subsequently by thin layer chromatography to separate DNB from possible metabolites. Although only 10% of the applied dose was metabolized, the appearance of metabolites was found to vary with dose and time. The major metabolites detected were

m-nitroacetanilide and *m*-nitroaniline. Addition of these compounds to the culture system did not result in any evidence of toxicity at concentrations up to 10^{-4} M. A similar profile of metabolite production was observed with Sertoli cell only cultures. Further metabolic studies conducted *in-vitro* by Foster *et al.* (1987a), again using 14 C-labelled *m*-DNB, showed that both Sertoli+germ cell co-cultures and Sertoli cell only cultures were capable of xenobiotic metabolism with nitroreduction of DNB being the principal metabolic route (Figure 5.1). It was postulated therefore that DNB or a Sertoli cell metabolite (probably an intermediate of nitroreduction produced *in-situ*) was responsible for the testicular damage observed following administration of the compound *in-vivo* (for review see Foster 1989). In support of this hypothesis, Cave and Foster (1990) found that the toxic effects of *m*-DNB and 3-nitrosonitrobenzene (a reduced intermediate of *m*-DNB) could be exacerbated by diethyl maleate (which depletes glutathione levels) and ameliorated by scavengers of reactive intermediates, such as cysteamine and ascorbate.

A direct comparison was made of the ability of the three isomers (ortho, meta and para) of DNB to induce testicular damage in the adult rat following a single oral dose of the compound (Blackburn *et al.*, 1988). Both meta- and para-DNB produced cyanosis and splenic enlargement when administered at a dose of 50mg/kg. Of the three isomers, only meta-DNB produced significant decreases in testicular weight and resulting histological changes in the seminiferous epithelium. In a subsequent study, the pathogenesis of testicular damage resulting from a single oral dose of 5, 10, 15 or 25mg *m*-DNB/kg was studied in adult rats, using electron microscopy, at 6, 12, 24, 48 and 96 hours after dosing. At 12 hours after dosing, 25mg/kg *m*-DNB produced stage-specific lesions confined to stages VIII-XI of the spermatogenic cycle. Vacuolation and retraction of the Sertoli cell cytoplasm was observed in the region of primary spermatocytes. By 24 hours after administration of *m*-DNB, widespread Sertoli cell damage was evident and, in some tubules, this was associated with loss/degeneration of pachytene spermatocytes. Ultrastructural examination at this time confirmed that there were effects on Sertoli cells in the absence of germ cell damage. Similar effects were seen 48 hours after a single oral dose of 48 mg/kg *m*-DNB. Doses of 5 or 10mg/kg were without effect on the testis.

The conclusion that can be drawn from these studies is that the Sertoli cell is probably the initial target for the toxic action of *m*-DNB, with germ cell damage a secondary event. Although histological evidence provides a means

Figure 5.1. Metabolism of *m*-DNB in Sertoli cells, showing metabolites identified and proposed route, through possible reactive intermediates, which may result in toxicity. (Reprinted from Foster *et al.* 1987).



by which the initial lesion can be identified following disruption of spermatogenesis, it is difficult to assess accurately the degree of long term disruption and the rate of reversibility per animal using histological evaluations alone. An evaluation of sperm output over time following toxicant exposure permits the study retrospectively of testicular damage and of the recovery of spermatogenesis, it is possible to ascertain which cell types are affected initially following m-DNB exposure. Linder *et al.* (1986) showed that subchronic exposure to m-DNB by oral gavage decreased sperm production at a dose of 1.5mg/kg/day and rendered male rats infertile at 3mg/kg/day. In a subsequent experiment both acute and long-term effects following a single oral dose of m-DNB (48mg/kg) on sperm quality, quantity and fertilizing ability in the rat were assessed (Linder *et al.*, 1988). Testicular and epididymal parameters indicated that the compound produced a rapid decrease in testicular weight and sperm numbers, poor sperm quality and loss of fertilizing capacity post-treatment. Normal fertilizing ability was restored in most animals 13 weeks after exposure to m-DNB, whilst in others little or no recovery of testicular damage was evident. Further studies by Foster (1989) have shown a significant reduction in the percentage of females becoming pregnant after mating with males exposed to m-DNB at concentrations of 5 and 10mg/kg/day for 5 days. Complete recovery was, however, evident in both groups 63 days after treatment. Species differences have been observed, with rats more sensitive than mice, following a single oral dose of m-DNB (Evenson *et al.*, 1989). In addition, young mice and rats appear to be more resistant to the effects of m-DNB than older mice and rats (Evenson *et al.*, 1989; Linder *et al.*, 1990).

Although it is recognized that m-DNB has a direct effect on the seminiferous epithelium, several studies have suggested that such degenerative changes might, in turn, alter the hypothalamic-pituitary gonadal axis. Rehnberg *et al.* (1988) evaluated the endocrine status of male rats killed at 1, 7 and 14 days following a single oral dose of 32mg m-DNB/kg. Results of the study revealed that levels of FSH in serum were increased only at 14 days post-treatment. Significant increases in testosterone levels in IF and seminiferous tubule fluid were observed 7 and 14 days post-treatment, whereas a transient decrease in serum testosterone levels was noted at 1 day post-treatment. These results are in contrast to those obtained by Allenby (1990) who, in a similar experiment, evaluated the endocrine status of male rats (killed at various time points) following a single dose of 25mg m-DNB/kg. This study reported a time-dependent increase in the serum

levels of FSH and testosterone within 24 hours of dosing. The discrepancy between the results of Rehnberg *et al.* (1988) and Allenby (1990) may be due to variations between different animals, in levels of testosterone.

Endpoints currently used in assessing toxic damage to the testis rely almost exclusively on monitoring changes in testicular weight, blood levels of reproductive hormones (LH, FSH and testosterone) and, in some instances, semen profiles, but all of these parameters are relatively insensitive, and can only detect gross damage to the testis several weeks after initial exposure to the toxicant. Furthermore, these parameters do not indicate the stage or the nature of the defects in spermatogenesis induced by toxicant exposure. As a result, more effort has been directed towards identifying sensitive markers of altered Sertoli cell function. Williams and Foster (1988) showed that *m*-DNB perturbed normal Sertoli cell biochemical function *in-vitro*, as judged by the measurement of two Sertoli cell products, lactate and pyruvate (Jutte *et al.*, 1981), both of which are essential for germ cell viability (Jutte *et al.*, 1983). Williams and Foster (1988) observed dose-related increases in concentrations of both lactate and pyruvate in the culture medium following *m*-DNB exposure, and a resulting decrease in the lactate : pyruvate ratio from control values (10:1). These decreases were a reflection of altered Sertoli cell function and differed from the effects produced by other Sertoli cell toxicants (for example, mono-2-ethylhexyl phthalate (MEHP)), reflecting possible differences in the aetiology of Sertoli cell damage induced by these compounds *in-vivo*. Studies using Sertoli cell+germ cell co-cultures and Sertoli cell only cultures, have also demonstrated the isomer specificity of *m*-DNB *in-vitro* (Williams and Foster, 1988); ortho and para-DNB did not induce any significant changes in the production of lactate and pyruvate after 24 hour incubation, whereas meta-DNB induced significant increases in both products. These biochemical changes are, therefore, specific to the meta isomer of DNB which has been shown to produce damage to Sertoli cells *in-vivo* and *in-vitro*. Whilst these findings are encouraging in terms of providing a sensitive 'marker' of altered Sertoli cell function and/or dysfunction induced by *m*-DNB *in-vitro*, their wider application is limited as it is not feasible to measure the change in levels of lactate and pyruvate *in-vivo*. These results highlight the pressing need for a non-invasive 'marker' of early toxicant-induced disruption of spermatogenesis which can be measured, preferably in peripheral blood.

With this in mind Allenby *et al.* (1991b) evaluated the potential usefulness of immunoactive inhibin as a potential biochemical marker of

testicular dysfunction, both *in-vitro* or *in-vivo*. They observed that addition of m-DNB to ST cultures at 10^{-5} or 10^{-3} M stimulated basal secretion of inhibin two- to four fold on days 1-3 of culture. At the same doses, m-DNB also enhanced the secretion of inhibin by Sertoli cell cultures. Moreover, exposure of adult rats *in-vivo* to m-DNB at levels equivalent to those which stimulated secretion of inhibin *in-vitro* resulted in increased levels of inhibin in testicular interstitial fluid at 1 and 3 days post-treatment. A similar increase in the levels of inhibin was observed following treatment with the closely-related chemical nitrobenzene. These findings demonstrate that early warning of adverse changes in spermatogenesis could be obtained by monitoring this protein, though its application to man is not so straightforward. This is because inhibin is (and inhibin-related peptides are) known to be produced by extratesticular sources (Meunier *et al.*, 1978) which probably limits its usefulness.

A more promising approach is to use 'markers' which emanate from the germ cells, as many of these are likely to be unique, thus avoiding the problems related to specificity associated with Sertoli cell-derived proteins. Reader *et al.* (1991) assessed the usefulness of two germ cell proteins, namely, lactate dehydrogenase (LDH-C4) a cytoplasmic enzyme involved in basic energy metabolism (but which is unique to meiotic and post-meiotic germ cells) and sorbitol dehydrogenase (SDH), an enzyme which converts sorbitol to fructose. In addition, two Sertoli cell products were monitored, namely leucine aminotransferase (LAT) and androgen-binding protein (ABP). Administration of a single oral dose of m-DNB to adult rats resulted in increased levels of ABP and LDH-C4 in peripheral blood for up to 14 days. No changes in either testicular or plasma SDH or LAT were observed. The results of this study are encouraging and suggest that LDH-C4 may be useful as an indicator of germ cell damage, unlike ABP, which is difficult to monitor, due to the fact that ABP is very similar immunologically to sex-hormone binding globulin (SHBG), which is secreted into the bloodstream by the liver in man (Cheng *et al.*, 1984).

Another non-invasive method of monitoring disruption of spermatogenesis was suggested by Moore *et al.* (1992), who assessed the levels of urinary creatinine following administration of a single dose of m-DNB to adult rats. Within 24 hours of dosing, increases in the level of urinary creatinine excretion and the urinary creatine : creatinine ratio were observed. Similar results were obtained following a single oral dose of MAA (a known germ cell toxicant) and di-n-penthyl phthalate (DDP) (a Sertoli cell

toxicant). The findings of this study suggest that creatinuria may be a useful non-invasive marker of early testicular damage, but they do not permit a conclusion to be made regarding the source of creatinine within the testis, since the toxicants used in this study work via different routes; *m*-DNB and DPP alter Sertoli cell function with germ cell damage occurring as a secondary event, whereas MAA is thought to act directly on pachytene spermatocytes (Foster *et al.*, 1983; Creasy *et al.*, 1985, 1987).

In summary, all known methods which induce disruption of spermatogenesis do so by causing initial lesions which are both stage- and cell-specific. The stage-specificity of germ cell degeneration after such treatments is probably an indication that only selected Sertoli cell+germ cell interactions are affected following the initial disruption of spermatogenesis.

It is generally believed that the Sertoli cell, which is intimately associated with the developing germ cells, orchestrates spermatogenesis, the process whereby stem cells are transformed into spermatozoa through a number of well-defined stages (Leblond and Clermont, 1952). It is now thought that the Sertoli cell controls spermatogenesis via the secretion of different proteins and that these vary cyclically according to the stage of the spermatogenic cycle (Parvinen, 1982, 1993). Furthermore, there is increasing evidence suggesting that germ cells are capable of modulating directly the secretory function of the Sertoli cells, according to their own specific requirements (Djakiew and Dym, 1988; Jégou 1991, 1992; Sharpe, 1993). It is presumed that some of these functions are either altered selectively or are more sensitive to the adverse effects of particular chemicals or other treatments.

As the various stages of the spermatogenic cycle can be isolated by transillumination-assisted microdissection (Parvinen *et al.*, 1986) it is potentially possible to identify these alterations at the protein level at various time points after toxicant exposure. Identification of these changes would (a) identify the possible biochemical cause(s) of the testicular toxicity of the chemical in question or, at worst, an early consequence of such toxicity, (b) give an insight into normal control mechanisms in spermatogenesis, and (c) identify proteins which have potential use as early markers of toxicant-induced abnormalities of spermatogenesis, provided that they are detectable in peripheral blood and that assays can be developed for their measurement (Sharpe, 1992).

The objective of the experiments described in this chapter was to assess whether reproducible stage-specific changes in the section of proteins by

isolated ST could be identified following exposure to *m*-DNB and, further, to equate these to temporal histological changes. Seminiferous tubule-secreted proteins were targetted specifically because current thinking suggests that Sertoli cell-germ cell interactions centred around secreted proteins (Sharpe, 1992).

5.2. Experimental Procedures

5.2.1. Reagents

Meta-dinitrobenzene (*m*-DNB) was obtained from Aldrich Chemical Co. (Gillingham, Dorset, UK) and was recrystallized from ethanol to greater than 99% purity as determined by gas chromatography. The vehicle for *m*-DNB administration was polyethylene glycol 600 (PEG 600) obtained from Sigma (Poole, Dorset, UK).

5.2.2. Dosing regime

Young adult male rats aged approximately 70 days were administered a single oral dose of *m*-DNB (50mg/kg bodyweight) by gavage in PEG 600 (1ml/kg). This dose of *m*-DNB has been shown previously to cause severe disruption of spermatogenesis *in-vivo* (Blackburn *et al.*, 1988).

5.2.3. Perfusion-fixation of control and *m*-DNB-treated rats

At 24 hours after *m*-DNB treatment, 2 rats were perfusion-fixed according to the methods described in Chapter 3 and data compared to data for 2 perfusion-fixed control rats.

5.2.4. Procedure for isolation of ST from control and *m*-DNB-treated rats

At 12, 24 and 72 hours after *m*-DNB treatment, seminiferous tubules were isolated from control and *m*-DNB-treated rats according to the methods described in Chapter 3.

5.2.5. Procedure for isolation of ST from control rats

Seminiferous tubules were isolated from untreated control rats and cultured for 24 or 72 hours in the presence or absence of *m*-DNB 10^{-4} M according to the methods described in Chapter 3.

5.2.6. Treatment of ST Cultures

m-DNB was added to cultures at a dose of 10^{-4} M in dimethyl sulphoxide (DMSO : BDH) at a final concentration of 0.3% (v/v), while controls received an equal volume of this vehicle. Treatments caused no appreciable alteration of the culture medium pH. The 35 S-methionine was added to cultures at the start of incubation and this, together with media and toxicants, was replaced every 24 hours when incubation was for a 72 hour period; media was analysed for protein changes after 24 and 72 hours of culture. The dose of 10^{-4} M m-DNB was chosen as being approximately equivalent to the peak levels which are achieved *in-vivo* following oral administration of 50mg/kg m-DNB (P.M.D. Foster - personal communication).

5.2.7. Measurement of newly synthesized proteins

Incorporation of 35 S-methionine into both secreted and intracellular proteins was determined by precipitation of aliquots of the culture medium or cell lysates with 10% (v/v) trichloroacetic acid, according to the methods described in Chapter 3.

5.2.8. Two-dimensional SDS PAGE

Newly synthesized 35 S-methionine labelled proteins secreted by isolated ST were evaluated by 2-D SDS PAGE according to the methods described in Chapter 3. ST conditioned medium from control and m-DNB-treated rats from the same experiment were always run in parallel on SDS PAGE, and subsequent determination of any change in the relative abundance of proteins on the gels was by comparison of gels from treated animals with their respective control from the same experiment. There was little variation between the gels from different experiments.

5.2.9. Collection of plasma and testicular IF

At 24 and 72 hours after m-DNB treatment, blood plasma and testicular interstitial fluid were collected from 5 control and 5 m-DNB-treated rats and stored for measurement of inhibin according to the methods described in Chapter 3.

5.3. Results

5.3.1. Effect of m-DNB on testicular morphology

Exposure of rats to a single oral administration of m-DNB caused rapid and extensive disruption of spermatogenesis. The most obvious effect of m-DNB on the testis at 24 hours post-treatment was the appearance of degenerating pachytene spermatocytes (Figure 5.2) and general disorganization of the seminiferous epithelium (Figure 5.2). This degeneration was found to be stage-dependent, occurring in tubules at stages VI-XIII of the spermatogenic cycle. However, round spermatids at stages I-VII also appeared abnormal in comparison to controls, although none were actually pyknotic.

5.3.2. Effect of m-DNB on testicular weight

Mean testicular weight in rats treated with 50mg/kg m-DNB was reduced significantly at 3 days ($p < 0.01$) but not at 1 day post-treatment (Figure 5.3).

5.3.3. Stage-dependent differences in overall protein secretion

The overall level of incorporation of ^{35}S -methionine into proteins secreted into the incubation medium by ST at stages VI-VIII from control rats was more than double that at stages II-V or IX-XII, as has been reported previously (Sharpe *et al.*, 1992), whereas incorporation into intracellular proteins showed no such difference between these stages (Figure 5.4). This difference was evident in the control group in each of the experiments described below. In view of this and differences between experiments in the actual level of incorporation of ^{35}S -methionine into ST-secreted proteins, data have been normalized by expressing incorporation as a percentage of the mean control value for that stage.

5.3.4. Effect of m-DNB on overall protein secretion

Following the administration of a single oral dose of m-DNB to rats there was a decrease in the incorporation of ^{35}S -methionine into newly synthesized proteins secreted into the incubation medium by ST at stages VI-VIII when isolated 12 hours after treatment. ST at stages II-V and IX-XII showed no change in incorporation. With ST isolated from rats 24 after treatment, there was a 26% increase in incorporation of radiolabel into secreted proteins at stages II-V, whereas ST at stages VI-VIII showed a significant decrease in incorporation ($p < 0.001$ in Figure 5.5). With ST isolated from rats 72 hours

Figure 5.2. Seminiferous tubules isolated at different stages of the spermatogenic cycle from control rats (top) showing pachytene spermatocytes (large arrowheads), round spermatids (small arrowheads) and the heads of elongate (small arrows) or rats pretreated with 50mg/kg *m*-DNB 24 hours earlier (bottom) showing degenerating pachytene spermatocytes (large arrowhead). Scale bar = 300 μ m.

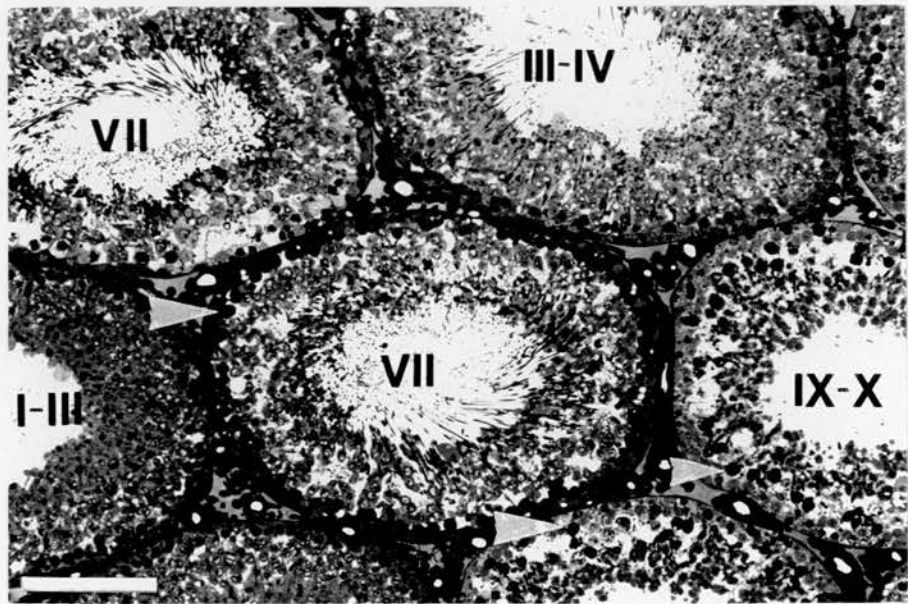
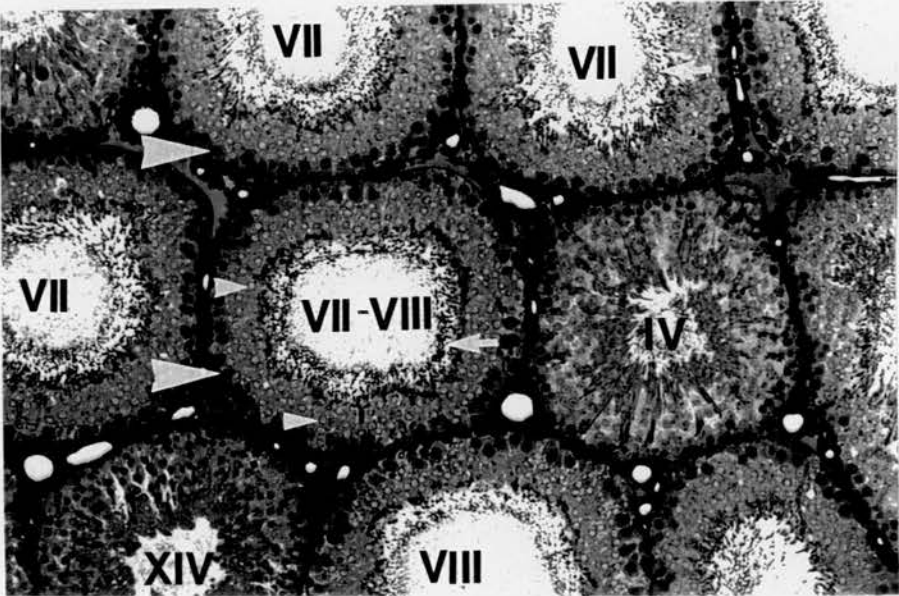
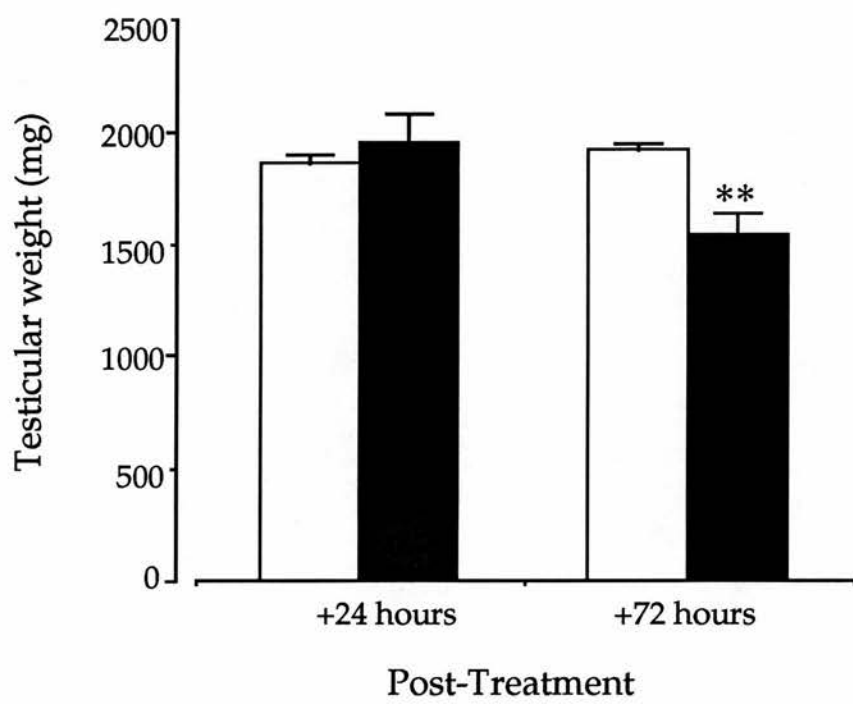


Figure 5.3. Effect of a single oral administration of *m*-DNB (50 mg/kg) (toned bars) or vehicle alone (1ml/kg) (open bars) on testicular weight at 24 or 72 hours post-treatment. Values are means \pm SD for 5 rats. ** $p < 0.01$, in comparison with respective control group.



after the administration of *m*-DNB, the decrease in incorporation of radiolabel into secreted proteins at stages VI-VIII was more pronounced when compared to controls (Figure 5.5). It was, however, impossible to distinguish stages II-V and IX-XII using the normal criteria (presumably because of degeneration of germ cells), which rely on changes in the density and the position of the nucleus of the elongate spermatids. To circumvent this problem, all stages other than VI-VIII were isolated. When these stages were compared with the appropriate pool of ST isolated from controls, incorporation of ^{35}S -methionine into secreted proteins was reduced drastically (Figure 5.5). ST that had been isolated from untreated control rats and cultured in the presence of *m*-DNB (10^{-4}M) for 24 hours exhibited changes in the incorporation of ^{35}S -methionine into secreted proteins (Figure 5.6) which were comparable to those observed for rats exposed to *m*-DNB for 24 hours *in-vivo*. When compared with controls, incorporation into secreted proteins at stages VI-VIII and IX-XII was reduced by 22% and 30%, respectively, after culture with *m*-DNB, whereas incorporation of ^{35}S -methionine was increased marginally, but not significantly, at stages II-V (Figure 5.6). After 72 hours in culture in the continuous presence of *m*-DNB, ST at stages VI-VIII and IX-XII still showed a significant decrease in incorporation of ^{35}S -methionine into secreted proteins over the last 24 hours of culture when compared with controls. ST isolated at stages II-V and cultured for 72 hours in the presence of 10^{-4}M *m*-DNB showed no change in incorporation (Figure 5.6).

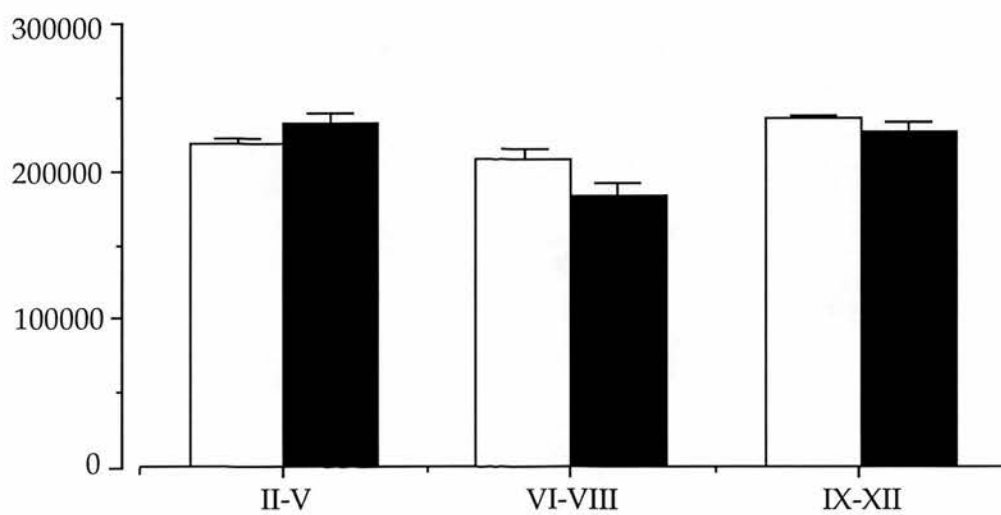
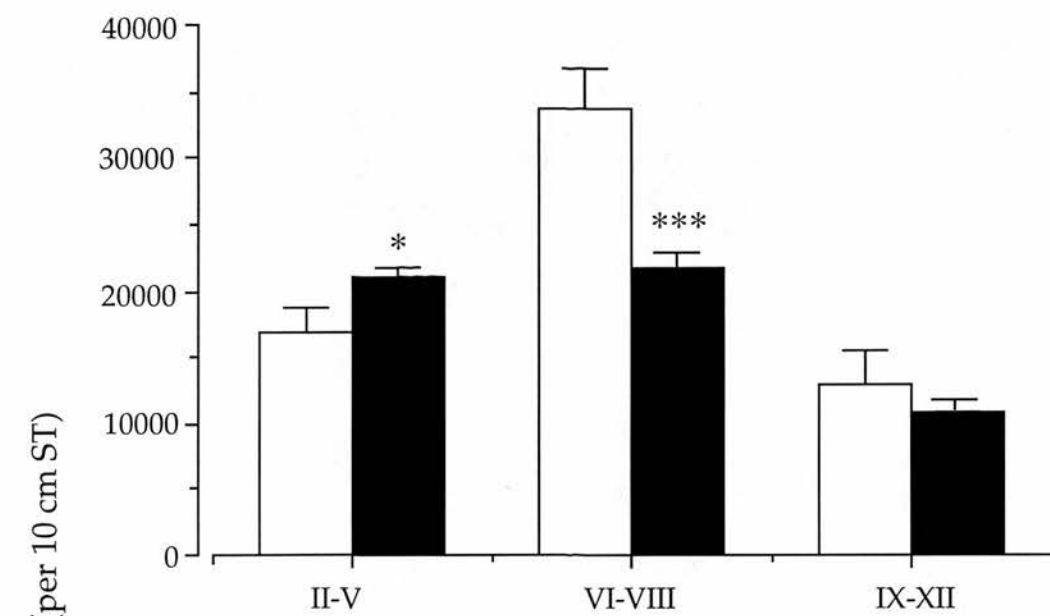
5.3.5. Effect of *m*-DNB on synthesis of intracellular proteins

Following treatment with *m*-DNB 12 or 24 hours earlier no significant change in incorporation of ^{35}S -methionine into intracellular proteins at any of the three stage groupings was observed whereas, by 72 hours post *m*-DNB treatment, a significant decrease in incorporation of ^{35}S -methionine into intracellular proteins was observed at stages IX-V, VI-VIII (Figure 5.7).

5.3.6. Two-dimensional SDS PAGE

Exposure to *m*-DNB *in-vivo* causes germ cell degeneration within 24 hours at stages VI-XIII but not at stages II-V (Blackburn *et al.*, 1988; Allenby, 1990). In order to identify whether changes in specific secreted proteins either precede or accompany germ cell degeneration, newly synthesized proteins, present in the culture medium of ST isolated at stages II-V, VI-VIII or IX-XII, were

Figure 5.4. Incorporation of ^{35}S -methionine over 24 hours into newly synthesized secreted (top) or intracellular (bottom) proteins by seminiferous tubules isolated at different stages of the spermatogenic cycle from control rats (open bars) or rats pretreated with 50mg/kg *m*-DNB (toned bars) 24 hours earlier. Values are means \pm SD for three rats per stage group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, in comparison with respective control group.



Stages of the spermatogenic cycle

Figure 5.5. Incorporation of ^{35}S -methionine over 24 hours into newly synthesized proteins secreted *in-vitro* by seminiferous tubules isolated at different stages of the spermatogenic cycle from control rats (open bars) or rats pretreated with 50mg/kg *m*-DNB (toned bars) either 12 (top), 24 (middle) or 72 (bottom) hours earlier. As ST at stages II-V and IX-XII were impossible to distinguish using the normal criteria after pretreatment with *m*-DNB 72 hours earlier, all stages other than VI-VIII were isolated and compared with the appropriate pool of ST isolated from control. Values are means \pm SD of $n=6$. At each stage, incorporation has been expressed as a percentage of the mean control value (dashed line) obtained during the same experiment. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, in comparison with respective control group.

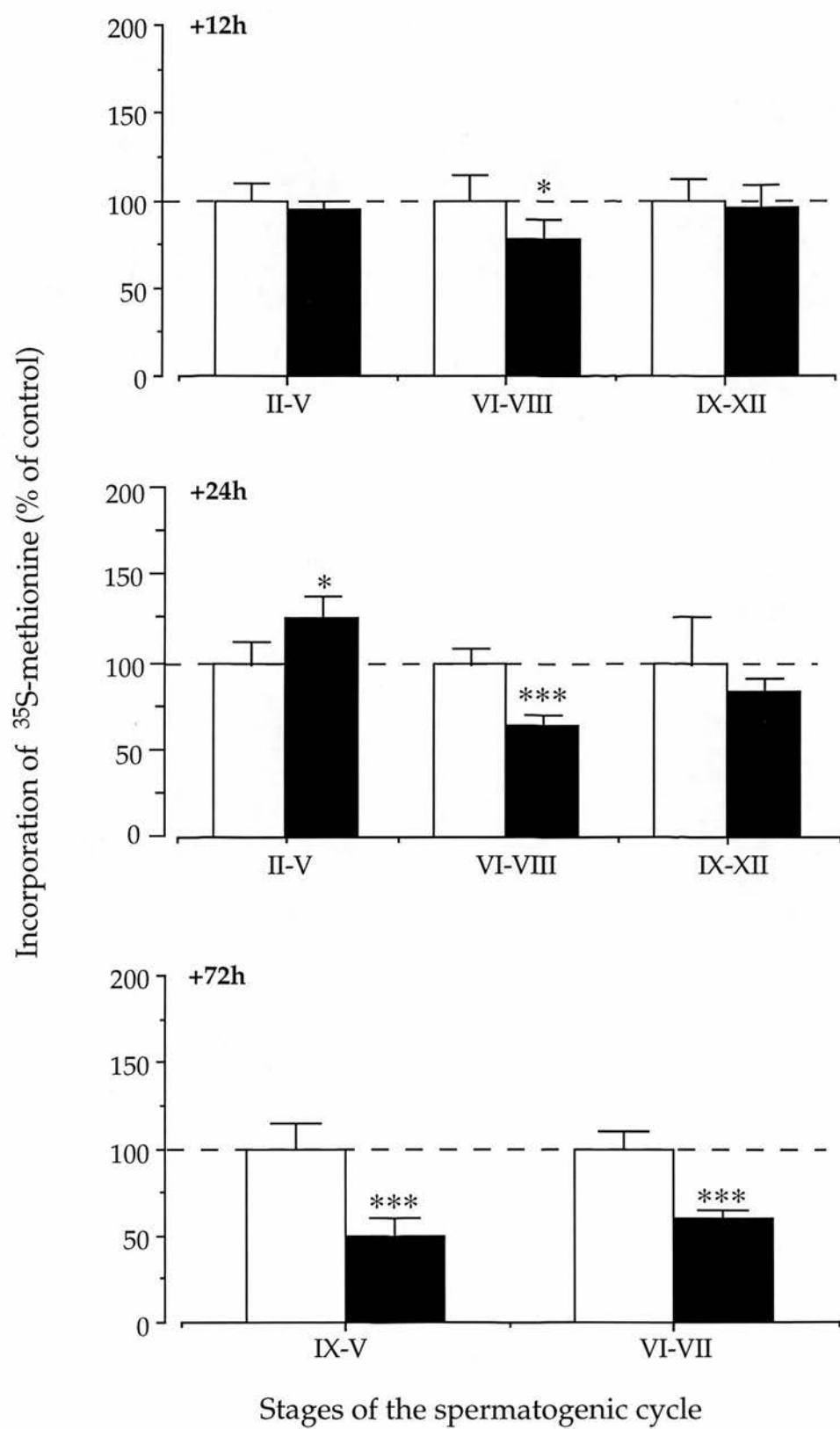
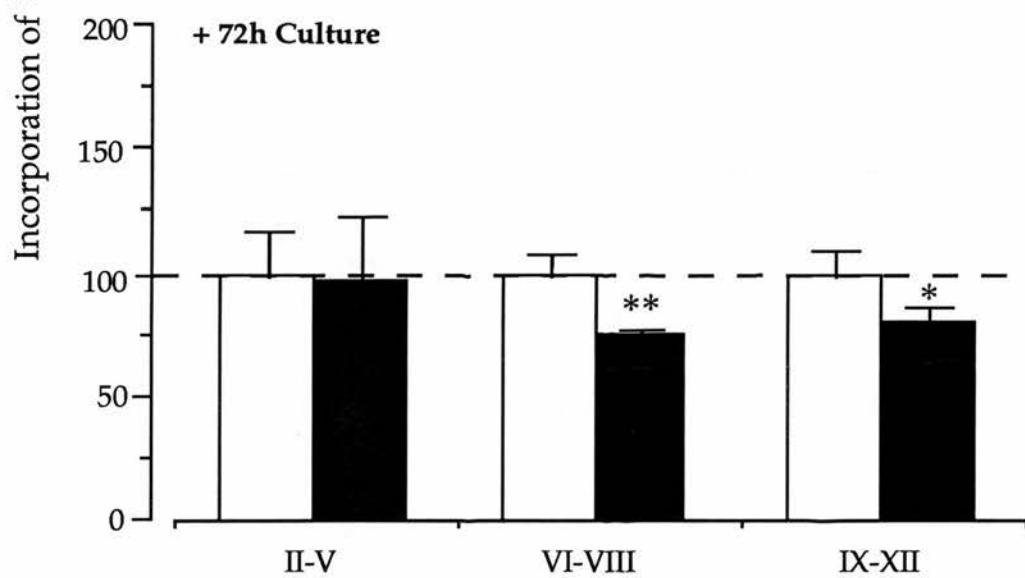
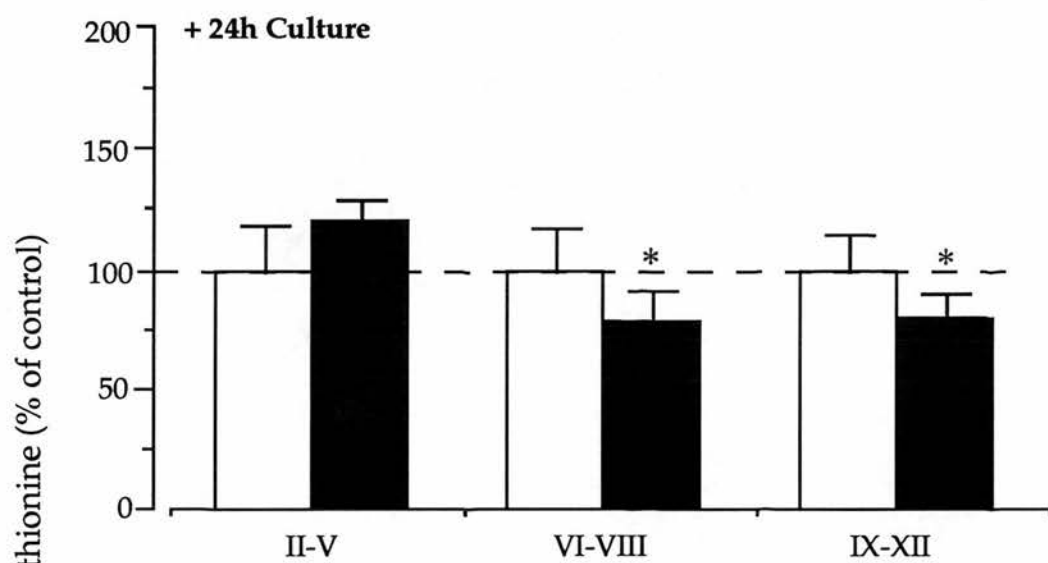
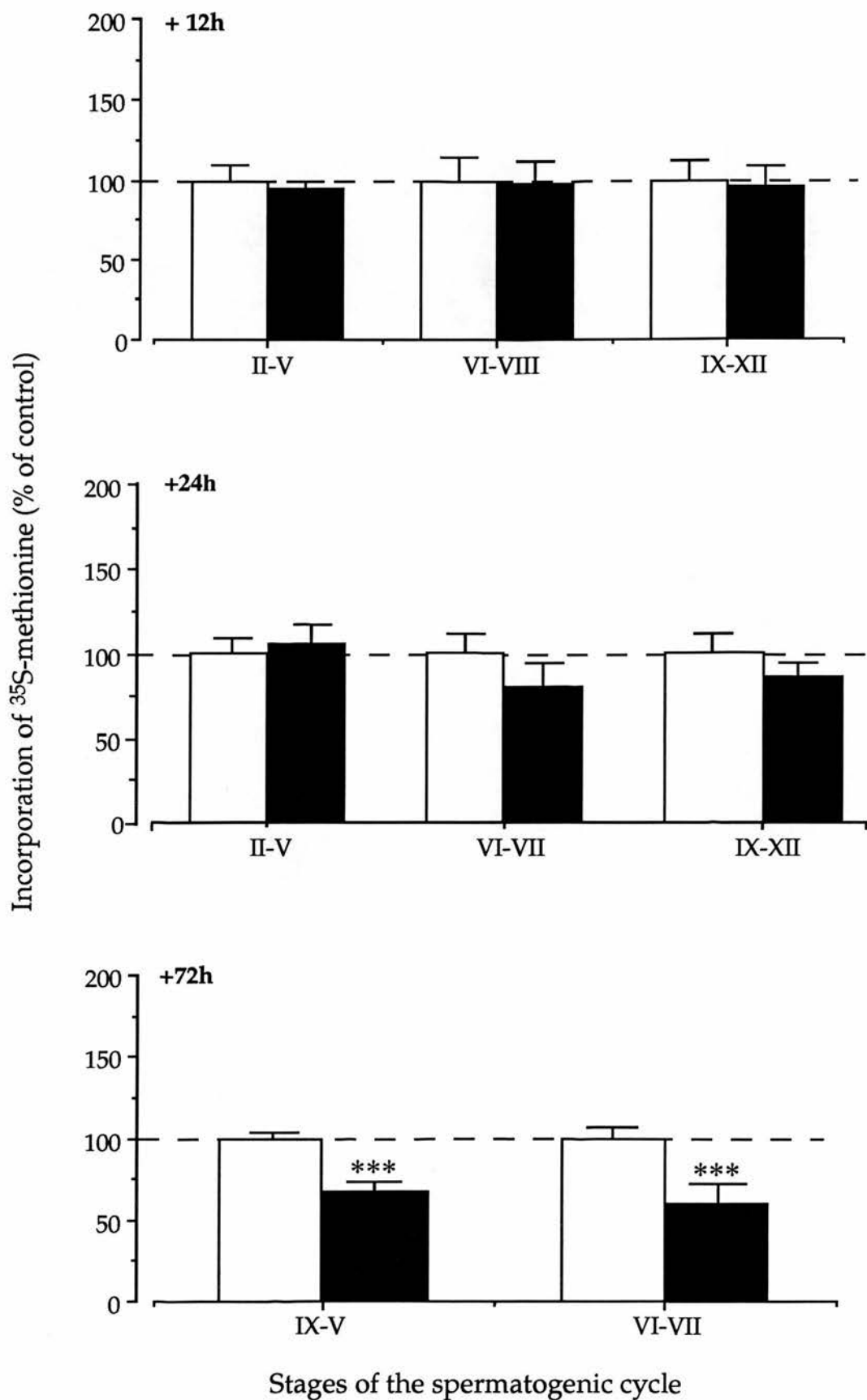


Figure 5.6. Incorporation of ^{35}S -methionine over 24 hours into newly synthesized proteins secreted *in-vitro* by seminiferous tubules isolated at different stages of the spermatogenic cycle from untreated control rats and then cultured in the absence (open bars) or presence (toned bars) of 10^{-4}M *m*-DNB for either 24 (top) or 72 (bottom) hours. At each stage, incorporation has been expressed as a percentage of the mean control value (dashed line) obtained during the same experiment. Values are means \pm SD of $n=4$. * $p<0.05$, ** $p<0.01$, in comparison with respective control group.



Stages of the spermatogenic cycle

Figure 5.7. Incorporation of ^{35}S -methionine over 24 hours into newly synthesized intracellular proteins by seminiferous tubules isolated at different stages of the spermatogenic cycle from control rats (open bars) or rats pretreated with 50mg/kg *m*-DNB (toned bars) either 12 (top), 24 (middle) or 72 (bottom) hours earlier. As ST at stages II-V and IX-XII were impossible to distinguish using the normal criteria after pretreatment with *m*-DNB 72 hours earlier, all stages other than VI-VIII were isolated and compared with the appropriate pool of ST isolated from controls. At each stage incorporation has been expressed as a percentage of the mean control value (dashed line) obtained during the same experiment. Values are means \pm SD of $n=6$. *** $p<0.001$, in comparison with respective control group.



analysed by 2-D SDS PAGE and visualized by fluorography.

The majority of radiolabelled proteins secreted by ST at stages II-V, VI-VIII or IX-XII were unaffected by exposure to *m*-DNB, but more than fifteen were found to have changed reproducibly. Of these proteins, ten were singled out as being potential markers of *m*-DNB-induced damage to spermatogenesis (numbered 1, 2, 3, 4, 5, 6, 7, 8, 9 and 13), on the basis that they showed marked changes in every experiment following exposure to *m*-DNB both *in-vivo* and *in-vitro*. A summary of the molecular weights and isoelectric points of these proteins, obtained from the analyses of four fluorograms, is given in Table 5 and the position of each is circled and labelled on each of the gels.

A comparison of the 2-D profile of proteins secreted by tubules from untreated rats at stages II-V, VI-VIII or IX-XII (Figures 5.8, 5.9 and 5.10) revealed that the secretion of several proteins was stage-dependent. The secretion of four of the identified potential marker proteins of *m*-DNB-induced damage (numbered 2, 6, 9, 13) is known to be stage-dependent, occurring predominantly at stages VI-VIII (the androgen-dependent stages). However, in the set of autoradiographs shown in this chapter (Figures 5.8, 5.9 and 5.10) the secretion of these four proteins was found at all stages isolated (II-V, VI-VIII, IX-XI), although, their secretion was found to be more prominent at stages VI-VIII (Figure 5.9). Following exposure to *m*-DNB, *in-vivo* or *in-vitro*, the secretion of these four proteins was reduced markedly. Four proteins (numbered 1, 4, 7, 8) were found to be secreted by ST at stages II-V, VI-VIII and IX-XII and, following exposure to *m*-DNB, either *in-vivo* or *in-vitro*, they showed pronounced reductions in abundance, or were completely absent. Proteins numbered 3 and 5 were of particular interest. The former existed as a prominent band of charge isomers of approximately 28-30 kilodaltons following *m*-DNB exposure and may represent high pI forms of SGP-2. Protein 5, which had a molecular weight of approximately 26 kilodaltons, was undetectable as a secretory product of normal adult ST, but could be induced by exposure to *m*-DNB *in-vitro* and *in-vivo* (Figures 5.8, 5.9, 5.10 and 5.11).

Protein No	M _r (kDa)	pI	Effect of <i>m</i> -DNB on relative abundance	Stage-dependence of secretion	Possible Identity
1	63 - 72	5.6 - 5.8	Absent or Decrease	All stages	unknown
2	38 - 43	5.8 - 6.2	Decrease	VI-VIII	ARP-4 (Sharpe <i>et al.</i> , 1992)
3	27 - 32	5.8 - 6.9	Increase	All stages	Possible charge isomers of SGP-2
4	23 - 24	5.4 - 5.8	Absent or Decrease	All stages	unknown
5	20 - 27	5.8 - 6.3	Induces appearance of protein	All stages	unknown
6	25 - 30	6.9 - 7.4	Decrease	VI-VIII	ARP-3 (Sharpe <i>et al.</i> , 1992)
7	16 - 20	5.3 - 5.9	Decrease	All stages	unknown
8	15 - 20	5.9 - 6.4	Decrease	All stages	unknown
9	13 - 14.5	6.9 - 7.6	Decrease	VI-VIII	ARP-2 (Sharpe <i>et al.</i> , 1992)
13	~9.5	6.9 - 7.4	Decrease	VI-VIII	ARP-1 (Sharpe <i>et al.</i> , 1992)

Table 5. List of seminiferous tubule secreted proteins which showed repeatable differences in relative abundance following treatment *in-vivo* or *in-vitro* with *m*-DNB (50mg/kg). Protein numbers correspond to those shown in Figures 5.8, 5.9, 5.10 and 5.11.

Figure 5.8. Incorporation of ^{35}S -methionine into newly synthesized proteins secreted *in-vitro* by seminiferous tubules isolated at stages II-V from control rats (top) or from rats treated 24 hours earlier with *m*-DNB (50mg/kg) (bottom). Proteins which change in relative abundance following *m*-DNB exposure are circled and numbered. The positions of the three major Sertoli cell secreted proteins, sulphated glycoprotein-1 (SGP-1), sulphated glycoprotein-2 (SGP-2) and cyclic protein-2 (CP-2), are indicated for reference.

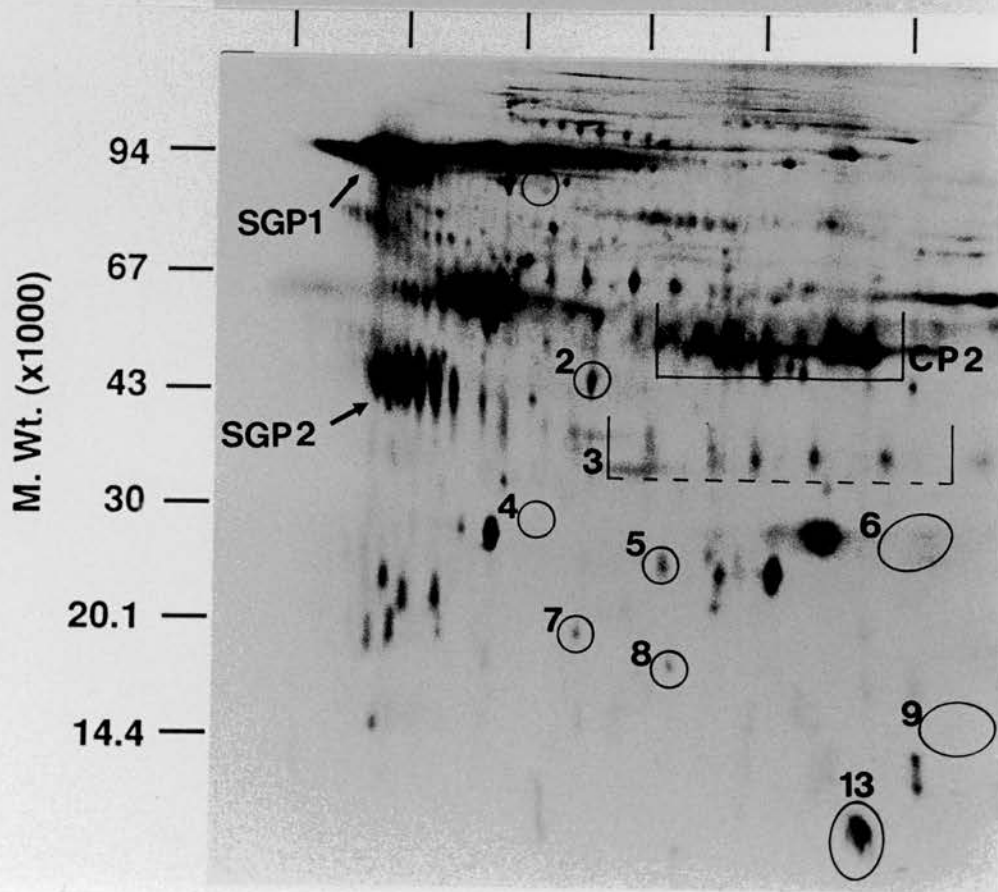
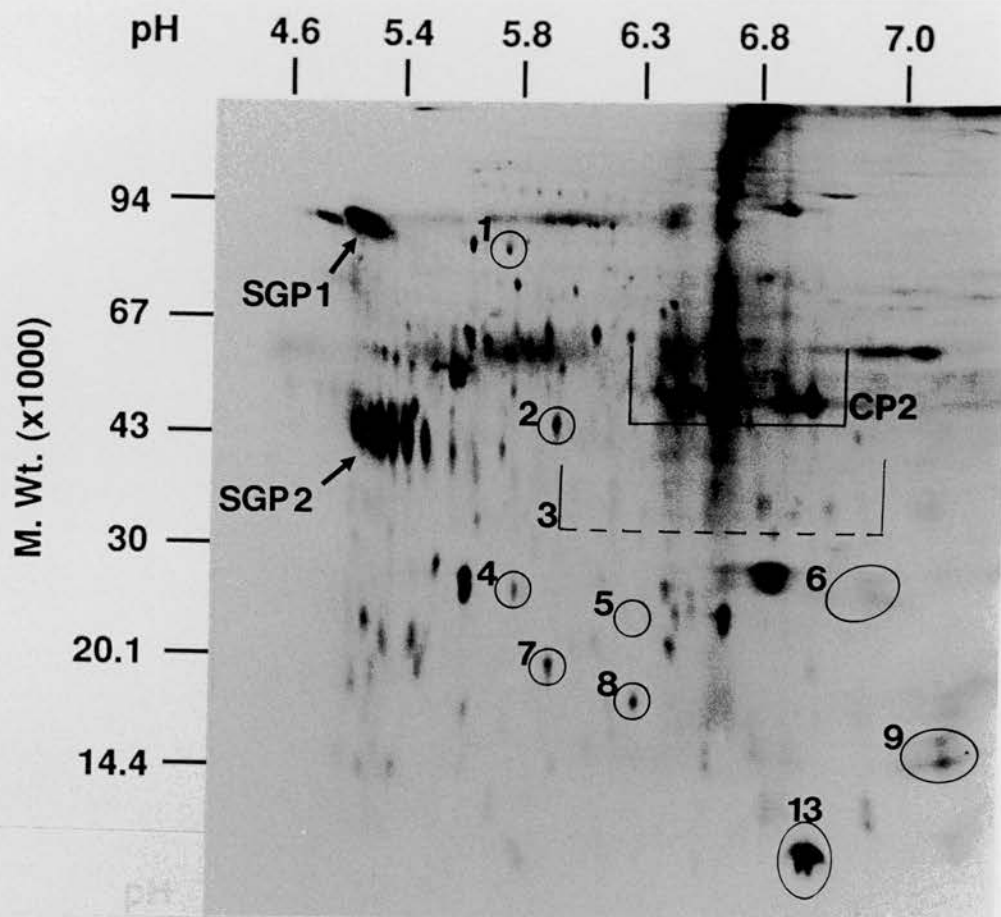


Figure 5.9. Incorporation of ^{35}S -methionine into newly synthesized proteins secreted *in-vitro* by seminiferous tubules isolated at stages VI-VIII from control rats (top) or from rats treated 24 hours earlier with *m*-DNB (50mg/kg) (bottom). Proteins which change in relative abundance following *m*-DNB exposure are circled and numbered. The positions of the three major Sertoli cell secreted proteins, sulphated glycoprotein-1 (SGP-1), sulphated glycoprotein-2 (SGP-2) and cyclic protein-2 (CP-2), are indicated for reference.

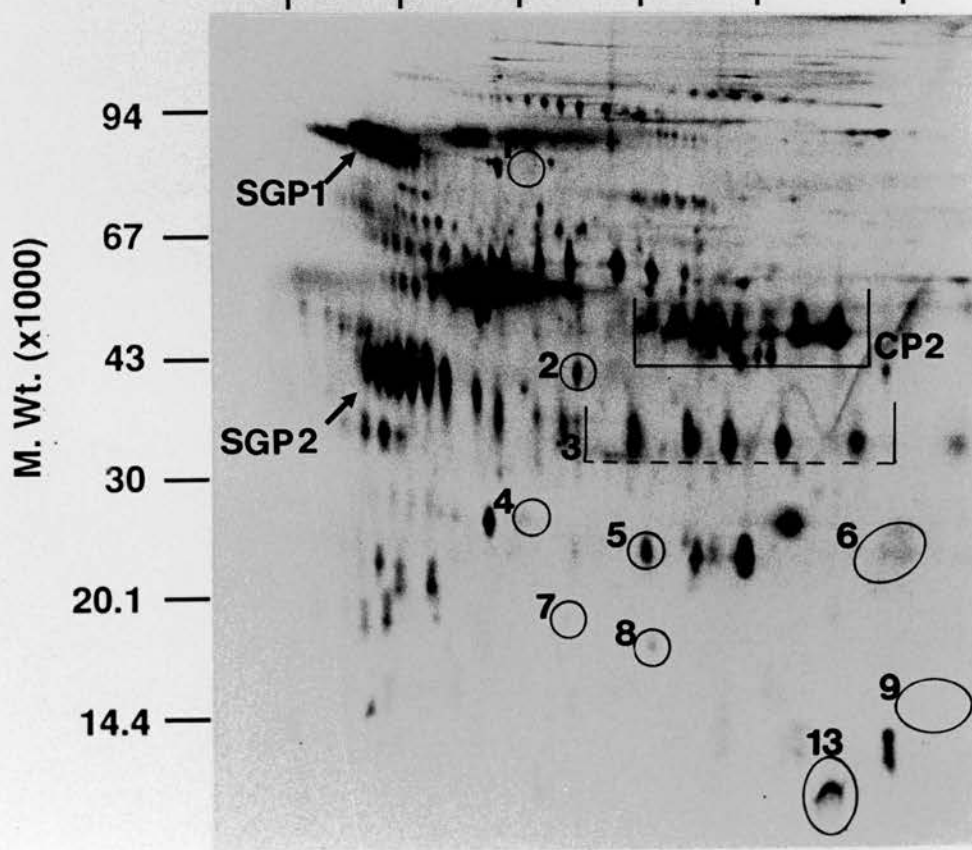
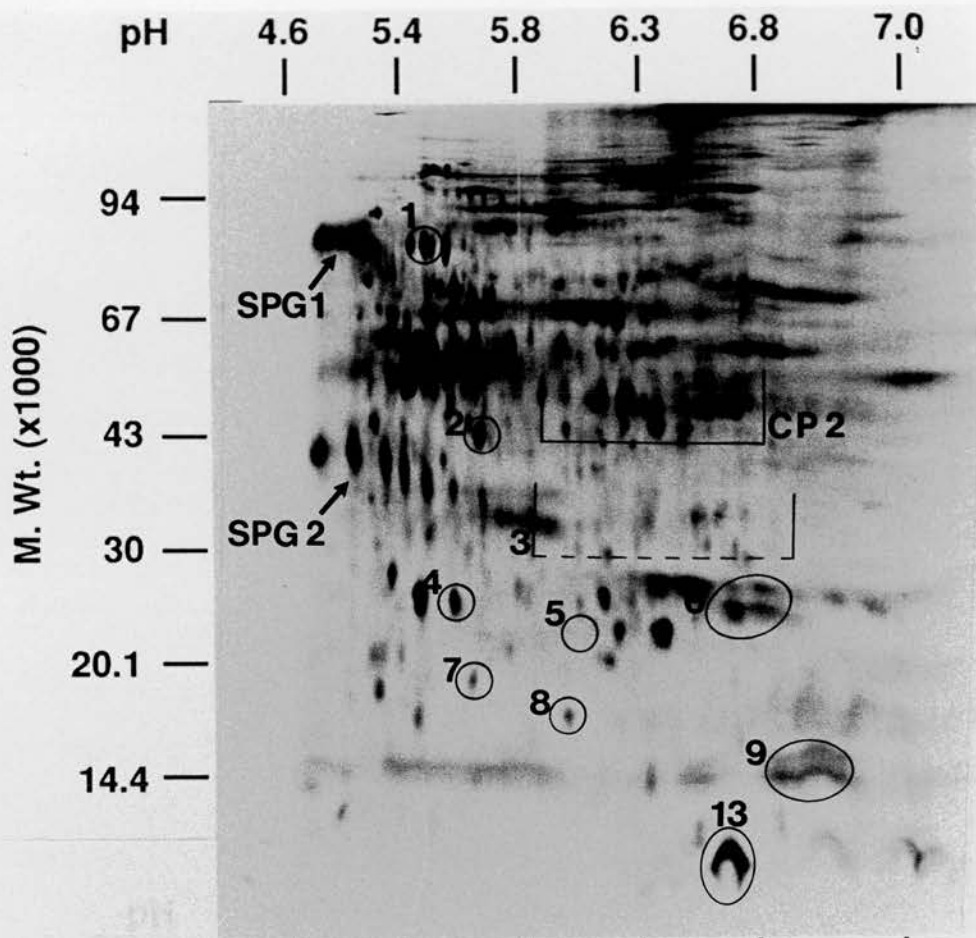


Figure 5.10. Incorporation of ^{35}S -methionine into newly synthesized proteins secreted *in-vitro* by seminiferous tubules isolated at stages IX-XII from control rats (top) or from rats treated 24 hours earlier with *m*-DNB (50mg/kg) (bottom). Proteins which change in relative abundance following *m*-DNB exposure are circled and numbered. The positions of the three major Sertoli secreted proteins, sulphated glycoprotein-1 (SGP-1), sulphated glycoprotein-2 (SGP-2) and cyclic protein-2 (CP-2), are indicated for reference.

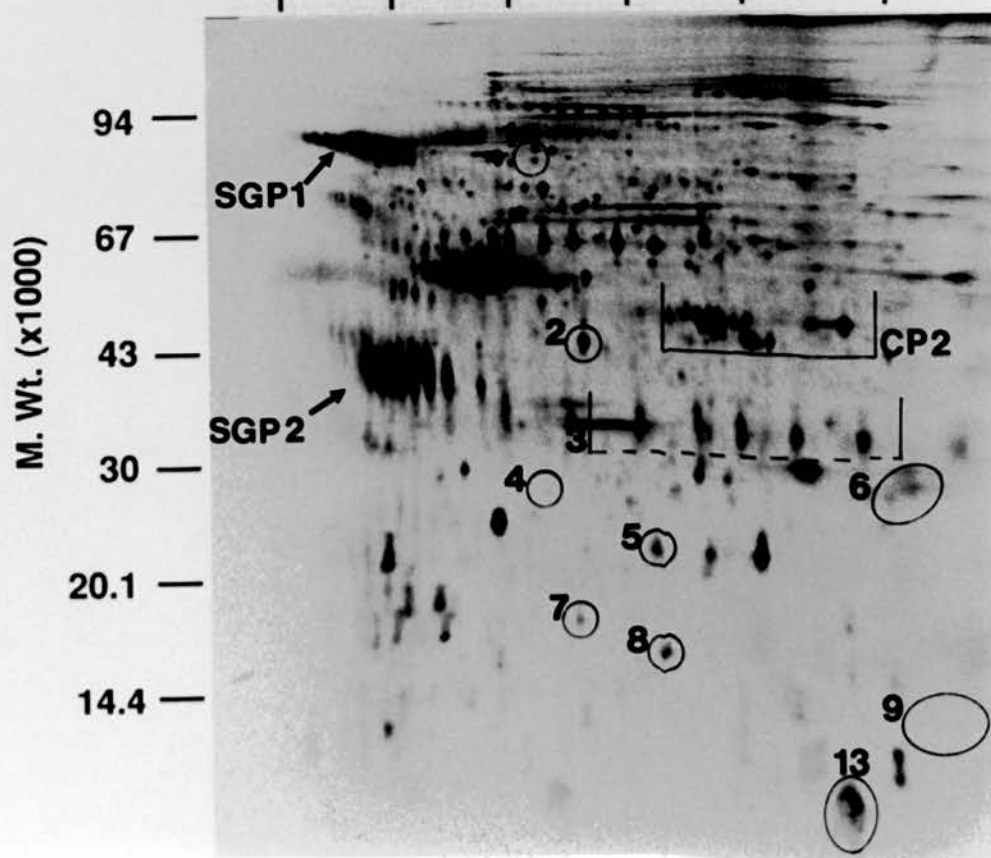
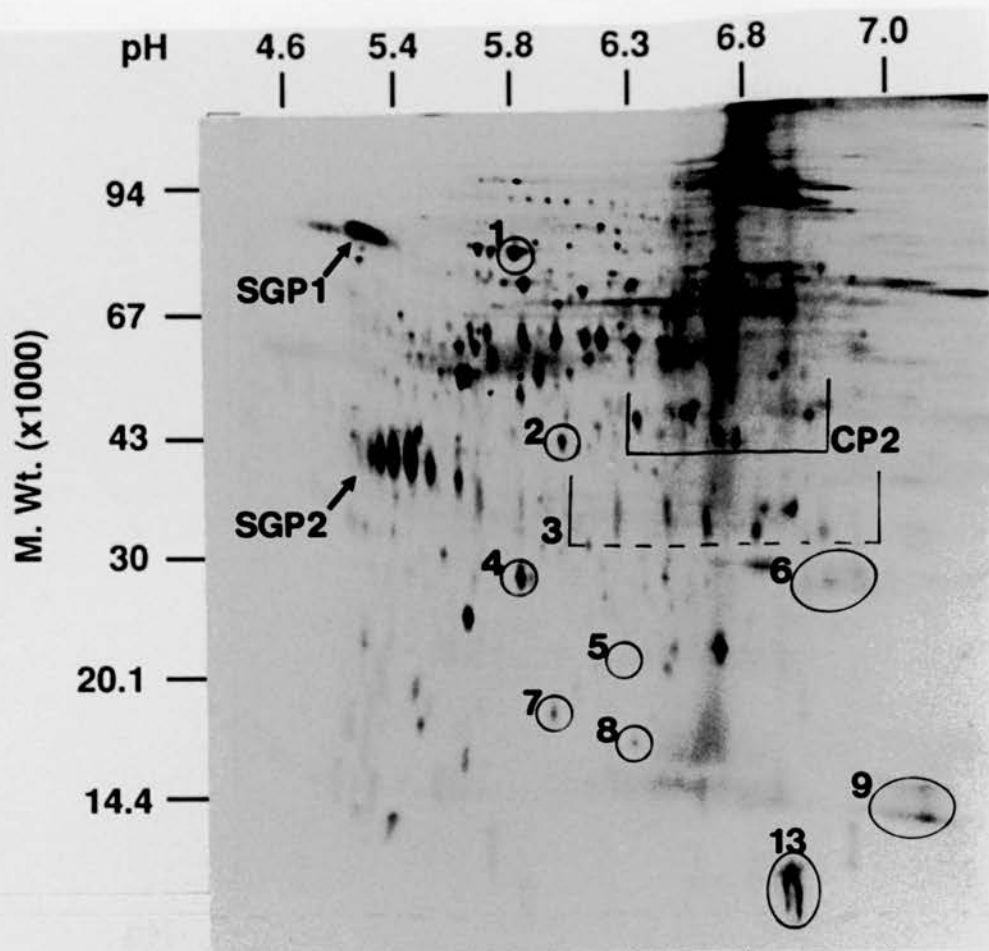


Figure 5.11. Incorporation of ^{35}S -methionine into newly synthesized proteins secreted *in-vitro* by seminiferous tubules isolated at stages VI-VIII from untreated control rats and cultured either in the absence (top) or presence (bottom) of *m*-DNB (10^{-4}M) for 24 hours. Proteins which change in relative abundance following *m*-DNB exposure are circled and numbered. The positions of the three major Sertoli cell secreted proteins, sulphated glycoprotein-1 (SGP-1), sulphated glycoprotein-2 (SGP-2) and cyclic protein-2 (CP-2), are indicated for reference.

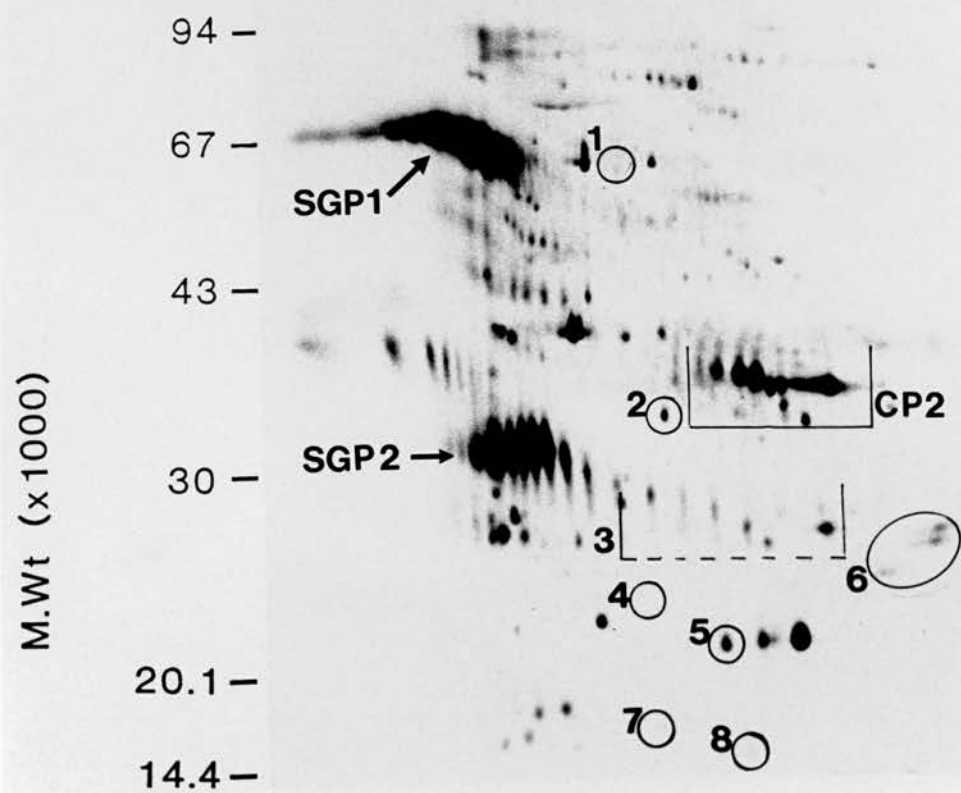
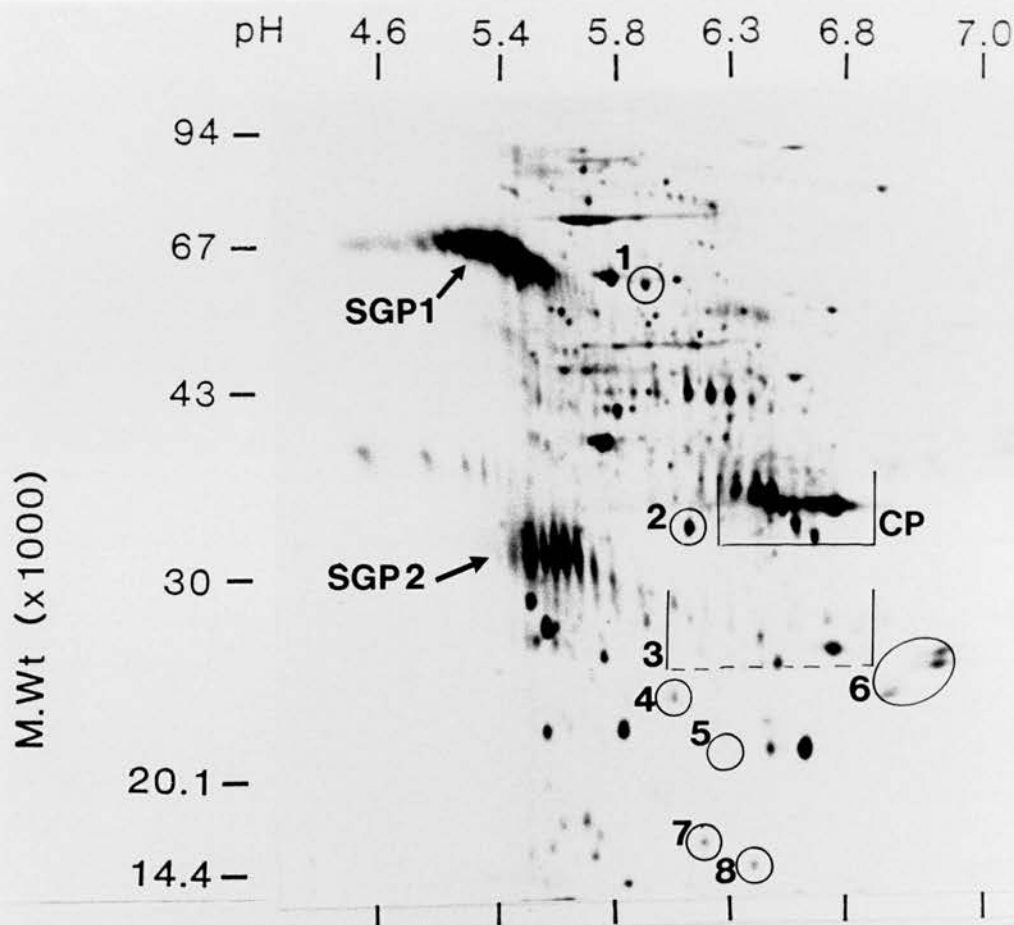
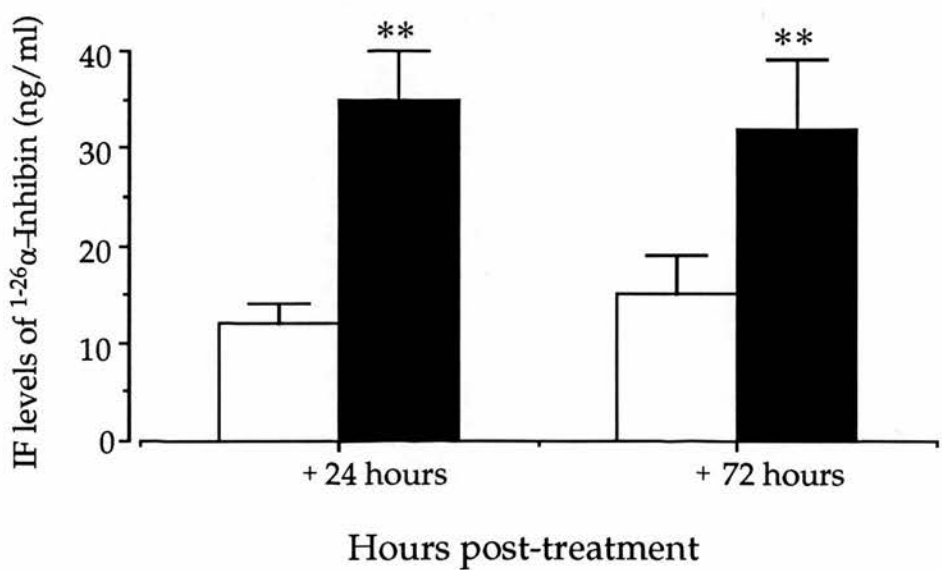
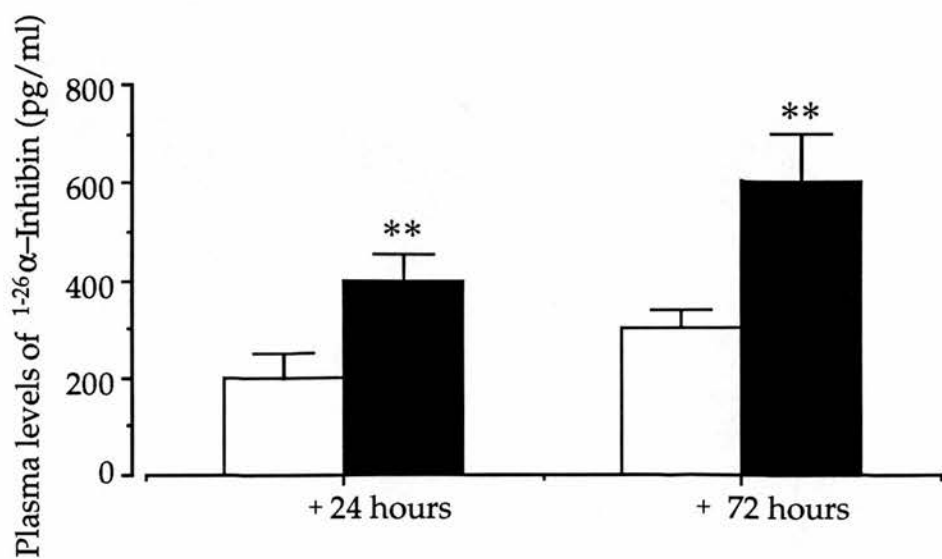


Figure 5.12. Effect of a single oral administration of *m*-DNB (50mg/kg) (toned bars) or vehicle alone (1ml/kg) (open bars) on levels of immunoactive inhibin in plasma (top) or testicular interstitial fluid (IF) (bottom) collected from rats at 24 or 72 hours after administration. Values are means \pm SD for 5 rats. ** $p < 0.01$, in comparison with respective control group.



5.3.7. Effect of m-DNB on levels of immunoactive inhibin

The levels of immunoactive inhibin in plasma testicular interstitial fluid (IF) were increased significantly at 1 and 3 days after treatment with m-DNB, when compared to the levels in control rats (Figure 5.12), although there was considerable variation between experiments in the levels of immunoactive measured in IF and plasma. However, irrespective of this variation, administration of m-DNB caused a 2- to 3-fold increase in the secretion of immunoactive inhibin, which was statistically significant.

5.4. Discussion

The objective of the experiments described in this chapter was to identify potential biochemical markers of early toxicant-induced disruption of spermatogenesis, by evaluating changes in the secretion of specific proteins from isolated seminiferous tubules following exposure to m-DNB.

m-DNB is a known testicular toxicant *in-vivo* (Cody *et al.*, 1981; Blackburn *et al.*, 1985, 1988; Foster *et al.*, 1986; Linder *et al.*, 1986, 1988; Rehnberg *et al.*, 1988; Hess *et al.*, 1988; Allenby *et al.*, 1990; Reader *et al.*, 1991). Studies by Foster *et al.* (1986, 1987), Lloyd and Foster (1987) and Williams and Foster (1988) have shown that m-DNB causes dose-dependent increases in the secretion of lactate and pyruvate by Sertoli cells *in-vitro*. Both lactate and pyruvate are required for normal germ cell function (Jutte *et al.*, 1981, 1983). In the present study, a single oral administration of m-DNB at a dose level known to cause severe testicular disruption (Blackburn *et al.*, 1988), and shown in this study to cause similar morphological change, resulted in stage-specific changes in the overall incorporation of ^{35}S -methionine into proteins secreted by ST isolated at 12, 24 or 72 hours after *in-vivo* treatment with m-DNB (Figure 5.5). Furthermore it was found that, when ST at the same stages (II-V, VI-VIII or IX-XII) were isolated from untreated control rats and cultured for 24 or 72 hours in the presence of m-DNB (10^{-4}M), a dose equivalent to the calculated peak testicular concentration achieved *in-vivo* following a single oral dose of 50mg/kg bodyweight (P.M.D. Foster – personal communication), there were stage-specific changes in incorporation of ^{35}S -methionine into ST-secreted proteins which were broadly comparable to those seen following exposure of rats to m-DNB *in-vivo* (Figure 5.6). These results provide biochemical confirmation of the present and previous morphological studies showing that a single oral administration of m-DNB

results in stage-specific disruption of spermatogenesis as determined morphologically (Blackburn *et al.*, 1988; Hess *et al.*, 1988; Allenby, 1990).

In this study the morphological effects of a single oral administration of 50mg/kg *m*-DNB on the rat testis were identified at the histological level within 24 hours of treatment. These initial effects were seen as vacuolation of the epithelium, coupled with the loss/degeneration of pachytene spermatocytes in seminiferous tubules at stages VII-XII of the spermatogenic cycle. In association with pachytene spermatocyte degeneration, many round spermatids at stages I-VII appeared abnormal in comparison to controls. These morphological effects are consistent with the previously reported findings identified in the rat by Blackburn *et al.* (1985, 1988), Hess *et al.* (1988) and Allenby (1990). The mechanism, by which *m*-DNB causes initial lesion(s) which are both stage- and cell-specific, are unknown, but exposure to other chemicals such as glycol ethers (Creasy and Foster, 1984), NB (Bond *et al.*, 1981; Allenby, 1990 and see Chapter 6), an imidazole compound (De Martino *et al.*, 1975) and other treatments such as local heating (Chowdhury and Steinberger, 1970; Bartlett and Sharpe, 1987 and see Chapter 8) or testosterone withdrawal (Sharpe *et al.*, 1990; Kerr *et al.*, 1993) have all been shown to cause degeneration of pachytene spermatocytes at specific stages of the spermatogenic cycle.

The changes observed in the incorporation of ³⁵S-methionine into overall proteins at stages VI-XII following treatment with *m*-DNB 12 hours earlier precede germ cell degeneration. However, by 24 hours post-treatment, pachytene spermatocytes at these same stages either appear pyknotic or have degenerated. Given that the protein changes seen at 12 hours post-treatment precede any morphological change, it may be suggested that the protein changes seen at stages VI-XII predict the future degeneration of germ cells at 24 hours post-treatment, at these same stages. The reason for the stage-dependent changes in protein secretion (particularly at stages VI-VIII) following *m*-DNB treatment is as yet unknown. It has been shown that mid-pachytene spermatocytes (i.e. those at around stages VI-VIII) have the highest rate of RNA synthesis and, perhaps, protein synthesis, of any of the germ cell types present in the seminiferous tubule (Monesi, 1965; Söderström and Parvinen, 1976). The results presented in this chapter show that *m*-DNB induced depletion of pachytene spermatocytes, had the most pronounced effect on protein secretion at stages VI-VIII, but had less dramatic effects at earlier or later stages.

It cannot be concluded, however, that pachytene spermatocytes are primarily responsible for the decrease in overall protein secretion by ST at stages VI-VIII, for several reasons. Firstly, isolated pachytene spermatocytes have a low level of protein secretion (Sharpe and McKinnell, unpublished data). Secondly, it has been shown that MAA-induced depletion of round spermatids can also decrease protein secretion by ST at stages VI-VIII (Sharpe *et al.*, 1993) and, thirdly, the decrease in protein secretion following treatment with m-DNB was observed prior to any morphological changes (i.e. prior to degeneration of pachytene spermatocytes). Taken together, results such as these suggest that the initial decrease in protein secretion is due to a primary change in the secretory function of the Sertoli cell, to such an extent that it can no longer maintain the viability of pachytene spermatocytes.

Analysis of ST-secreted proteins by 2-dimensional SDS PAGE identified more than fifteen proteins which changed reproducibly following exposure to m-DNB. Of these proteins, ten were singled out for particular attention on the basis that they showed marked changes in every experiment following exposure to m-DNB either *in-vivo* or *in-vitro*. The possible identity of these 'marker' proteins was assessed by reference to previous studies which have analysed the secretion of proteins from isolated seminiferous tubules, Sertoli cells and peritubular cells using 2-D SDS PAGE (Kissinger *et al.*, 1982; Wright *et al.*, 1983; Shabanowitz *et al.*, 1986; Skinner *et al.*, 1988). The secretion of four 'marker' proteins (numbered 2, 6, 9 and 13 in Figure 5.9) is known to be stage-specific and androgen regulated (Sharpe *et al.*, 1992), in addition to being germ cell dependent (McKinnell and Sharpe, 1992). In this study, the secretion of these proteins was reduced markedly at stages VI-VIII following exposure to m-DNB. Moreover, this reduction occurred without any corresponding significant decrease in the circulating or intratesticular levels of testosterone according to studies by Allenby (1990). The identity of proteins 3 and 5 is of particular interest because their secretion was found to increase at all stages in the absence of germ cells. The secretion of both of these proteins has been shown to be prominent in Sertoli cell+germ cell co-cultures from immature rats and then absent or of very low abundance in the adult (see Chapter 4). The identity of proteins 3 and 5 is unknown, though it is possible that protein 3 represents isomers of SGP-2 with a high pI. If this is true then it suggests that the degree of sulphation and/or glycosylation of SGP-2 (which probably determine its pI and M_r) is germ cell-regulated. Four proteins, numbered 1, 4, 7 and 8, were found to be secreted by ST at stages II-V, VI-VIII and IX-XII (Figures 5.8, 5.9, 5.10 and 5.11) and, following exposure

to m-DNB either *in-vivo* or *in-vitro*, they showed pronounced reductions in abundance or were completely absent. The secretion of proteins numbered 4 and 9 has been shown to decrease at a time when round spermatids are absent from the seminiferous tubule as a consequence of a single oral administration of the germ cell specific toxicant, methoxyacetic acid 18 days earlier (McKinnell and Sharpe, 1992). These results suggest either that proteins 4 and 9 are secretory products of round spermatids or that they are products of the Sertoli cells (or peritubular cells), the secretion of which is dependent on the presence of round spermatids. As m-DNB is a known Sertoli cell toxicant it is possible that, initially, a primary change in the secretory function of the Sertoli cell occurs which results in the loss/depletion of pachytene spermatocytes within 24 hours. The loss of these germ cells results, in turn, in secondary changes in Sertoli cell and/or germ cell secretory function, which subsequently affect round spermatids adversely. The fact that comparable protein changes were observed which either precede (stages II-V) or accompany (stages VI-XII) germ cell degeneration further supports this hypothesis.

The present study has also reassessed the usefulness of immunoactive inhibin as a marker of Sertoli cell function. Historically, levels of inhibin have been considered to vary inversely with the levels of FSH, in keeping with the negative feedback effects of inhibin on pituitary FSH secretion (de Jong and Robertson, 1985). The results obtained in this study have shown that one and three days after m-DNB treatment, coincident with the depletion of pachytene spermatocytes, levels of immunoactive inhibin in testicular IF and plasma were increased significantly when compared to controls (Figure 5.12). Similarly, blood levels of FSH have shown a similar pattern of change to inhibin levels following m-DNB exposure (Allenby, 1990). Taken together, results such as these suggest that blood levels of FSH and inhibin are related positively, which implies that the bioactivity of inhibin has been altered, following m-DNB exposure. The usefulness of inhibin as a biochemical marker is, however, somewhat limited, as the results obtained from experiment to experiment in this study were somewhat variable. The reason for this variable effect is unknown. In order for a compound, which is administered orally, to enter the circulatory system it must be absorbed by the gut. Previous investigators have demonstrated the necessity for intestinal gut flora in the metabolism and absorption of nitro aromatic compounds (Parke, 1961; Facchini and Griffith, 1981). In germ-free rats pre-treated with antibiotics the testicular toxicity of m-DNB was enhanced markedly, whilst

the haematological effects were removed. Therefore, it is possible that differences between animals in the intestinal gut flora and/or differences in the rate of absorption might alter the peak blood levels of m-DNB which, in turn, might account for the variability and lack of sensitivity in the measurements of immunoactive inhibin.

In conclusion, the experiments presented in this chapter have shown that m-DNB causes stage-specific changes in the secretion of proteins by isolated ST and has identified ten proteins which have potential use as markers of m-DNB induced damage to spermatogenesis. It is presumed that changes in these proteins may mediate some of the adverse testicular effects of m-DNB. Although the identity and precise role of these proteins remains unknown, the experiments presented in this chapter have shown that the monitoring of these proteins may be a viable and useful way of identifying early adverse changes to spermatogenesis.

6. Effect of nitrobenzene on ST protein secretion

Chapter 5 described the adverse effects of meta-dinitrobenzene on protein secretion by seminiferous tubules isolated from adult rats at different stages of the spermatogenic cycle. In this chapter the adverse effects of nitrobenzene are investigated in order to identify whether stage-specific changes in protein secretion occur which are similar to those obtained with the chemically related compound, meta-dinitrobenzene.

6.1. Introduction

Despite its relatively toxic nature, nitrobenzene (NB) has been used previously as a perfume and soap scent called 'essence of mirbane'. First synthesized by Mitscherlich in 1834, NB is used primarily today as a chemical intermediate (98%) in the synthesis of alanine. A small percentage of NB is also used in the production of dyes, solvents and propellants and in the manufacture of numerous nitroaromatic compounds, including meta-dinitrobenzene (see Chapter 5). In 1978, annual worldwide production of NB exceeded half a million tons with predicted increases of 5-10% per annum and with an estimated eight to twenty thousand tons being released annually into the environment as a result of the loss of NB through emission or release during manufacture or use (Dorigan and Hushon, 1981; Klappath, 1979).

NB poisoning in man has been recognized since at least 1856 (Chandler, 1919), with the majority of reported cases occurring following acute exposure by inhalation, or by dermal or oral routes (Gregory, 1906; Hamilton, 1919; Harrison, 1977). The most frequently reported consequence of exposure to nitrobenzene is methaemoglobinaemia. Nevertheless, considerable individual variation exists between the absorbed dose of NB and the severity of response in man. Experimentally induced methaemoglobinaemia due to nitrobenzene has been reported in experimental animals. In addition to its well-known capacity for production of methaemoglobinaemia in experimental animals and humans (Nabarro, 1948; Parkes and Neill, 1953; Magos and Sziza, 1959; Harrison, 1977), exposure to nitrobenzene also produced blood dyscrasia including decreases in erythrocyte and platelet counts and in circulating haemoglobin, haemolytic anaemia and bone marrow hyperplasia (Hamblin, 1949; Parkes and Neill, 1953). Beachamp *et al.* (1982) compiled a comprehensive review of the literature on NB toxicity. In this review, the potential target organs after acute and chronic NB administration in rats are the central and peripheral

nervous system (neuronal degeneration), hepatotoxicity with enlarged liver and altered serum chemistry, in addition to blood disorders.

Studies concerned with the potential reproductive effects of NB were initiated by Bond *et al.* (1981), who observed testicular lesions in Fischer 344 rats following high acute oral doses of NB (50-450mg/kg bodyweight). These testicular lesions were confined to the seminiferous tubule and consisted of dose- and time-dependent degeneration (vacuolation and enlargement) of primary and secondary spermatocytes, followed by necrosis of spermatogenic cells at 2 and 3 days after 450 and 300mg NB/kg bodyweight, respectively. Subsequently, multinucleated giant cells appeared in the place of the necrotic spermatogenic cells and there was a decrease in numbers of elongate spermatids in the seminiferous tubule, with resultant necrotic debris and a decrease in sperm number in the epididymis evident as early as 3 days after NB administration. There were, however, no apparent effects on the epididymal epithelium or on spermatogonia in the testis. Testicular lesions were evident only at doses of 200mg/kg and above and were produced consistently at 300mg/kg; they were not seen at doses between 75 and 160mg/kg. The LD₅₀ for orally administered NB in rats has been found to be 640mg/kg bodyweight (Fairchild, 1977).

The reversible nature of the NB-induced testicular degeneration in Fischer 344 rats was investigated by Levin *et al.* (1983), using sperm output as an index of spermatogenic disruption. This disruption was equated with histological examination of testes from rats treated identically in parallel experiments. Regular sequential measurements of sperm output were made using a non-invasive technique, whereby surgical anastomosis of the vas deferens to the urinary bladder was performed, such that daily urine samples could be collected in order to develop a profile of testicular damage per animal. The results of this study indicated that testicular damage resulting from a single oral administration of NB at 300mg/kg bodyweight was reversible so that sperm output was restored to near normal. Following NB treatment, lag periods of 26-30 days occurred before a reduced sperm output was observed. Between days 30-37, sperm were not present in the urine of treated rats, but thereafter there was a gradual recovery to 78% of control values by 76-100 days post-treatment. Full recovery was not obtained, possibly as a consequence of a limited number (approximately 10%) of tubules which were disrupted severely, and which failed to regenerate. Levin *et al.* (1983) concluded that restoration of spermatogenesis occurred only after cessation of exposure to NB and this suggests that undifferentiated

germ cells and spermatogonia are resistant to toxicity (Amann, 1982). The results of Dodd *et al.* (1987), discussed later, support this suggestion further.

Since NB is a mildly volatile liquid (0.28mm Hg at 25°C), NB is most likely to induce toxicity if inhaled. Hamm *et al.* (1984) exposed Fischer 344 and Sprague-Dawley CD rats or B6C3F1 mice to atmospheres of NB vapour at concentrations of 0, 5, 16 or 50ppm for 6 hours/day, 5 days/week for 90 days. At 50ppm, both strains of rats displayed bilateral degeneration of the seminiferous epithelium and a reduction or an absence of sperm in the epididymis, whilst the mice showed no signs of testicular lesions. Thus, the severity of the lesions was dependent upon strain and species differences (Medinsky and Irons, 1982). Some of these differences could be due to variations in the metabolism of nitrobenzene. Rickert *et al.* (1983) compared the metabolism in male Fischer 344 rats, Sprague-Dawley CD rats and B6C3F1 mice. In both rats and mice, urinary elimination of NB was the most important clearance route, with NB metabolites peaking at 6-24 hours after administration of ¹⁴C-NB (225mg/kg bodyweight) regardless of the route of administration. In both strains of rats the urinary metabolites after a dose of NB were p-hydroxyacetanalide, p-nitrophenol and m-nitrophenol. In addition to these metabolites Fischer 344 rats excreted one and Sprague-Dawley and CD rats two, very polar unidentified metabolites. B6C3F1 mice excreted the same metabolites as CD rats. In addition it was shown that mice excreted a substantial percentage of the dose (9.7%) as p-aminophenol sulphate, a compound that was not found in the urine of either strain of rat. The slow urinary excretion of NB is most likely due to slow metabolism of NB. The data from this study indicate species differences in the metabolism and excretion of nitrobenzene. In addition to species differences, the mechanism of NB-induced toxicity is complicated further by the influence of diet (Goldstein *et al.*, 1984). The presence of intestinal microflora is an important site for reductive metabolism due to the anaerobic environment in the intestine and the high activity of nitroreductase in the microflora, which is essential for the development of NB and m-DNB-induced methaemoglobinaemia (Reddy *et al.*, 1976; Facchini and Griffiths, 1981 and Chapter 5). However, this is only relevant in studies involving the oral administration of compounds. The final reduction products excreted in the urine are the result of intestinal flora activity; we know this because the urinary metabolites in NB-treated rats that have been pretreated with antibiotics to render them germ-free are different from those produced by rats which have a normal intestinal flora. It can be concluded therefore that the

metabolism of NB *in-vivo* is the result of a complex series of metabolic processes of both bacterial and mammalian origin (Levin and Dent, 1982).

Recently, reproduction and fertility evaluations have been performed by Dodd *et al.* (1987) in Sprague-Dawley CD rats following NB inhalation. In fertility evaluations, a two generation study revealed that exposure to NB at 40ppm for 6 hours/day for 5 days/week for 10 weeks resulted in a decrease in fertility of the Fo generation. The subsequent mature F1 male generation sired from the Fo male generation after a period of recovery also showed similar effects after NB exposure and, as with the Fo male generation, a time-dependent reversibility upon removal of NB was observed. Furthermore, Tyl *et al.* (1987) examined the potential for NB to induce developmental toxicity at target exposure concentrations identical to the study by Dodd *et al.* (1987). No *in-utero* effects of NB on testicular organogenesis were observed, although there was some maternal toxicity (spleen weight was increased slightly) evident at 10ppm, whilst at 40ppm bodyweight gain was reduced during gestational days 6-14, with absolute and relative spleen weights being increased by approximately 40%. In summary, the *in-vivo* experiments described above show that 1) NB is a testicular toxicant, 2) the rat is sensitive to NB-induced testicular effects, 3) the testicular effects are observed following either oral, inhalation and, possibly, dermal administration, and 4) the effects on the testes after acute exposure to NB are time-dependent and reversible.

Available evidence also indicates that NB, like m-DNB, is a Sertoli cell toxicant in view of its similar disruptive effects on various parameters of Sertoli cell function. Recently, Allenby *et al.* (1990) demonstrated that exposure of Sertoli cell and Sertoli cell+germ co-cultures to NB resulted in gross morphological changes, including Sertoli cell vacuolation and exfoliation of germ cells, in addition to producing dose-dependent increases in the secretion of three Sertoli cell products, namely, lactate, pyruvate and inhibin. Taken together, these results are indicative of a specific perturbation of Sertoli cell function *in-vitro*, and that disruption to spermatogenesis induced by NB *in-vivo* is therefore probably the result of a direct effect on the Sertoli cell. Further support for this interpretation is provided by the demonstration (Allenby, 1990) that *in-vivo* administration of NB results in stage-specific disruption to spermatogenesis similar to that induced by m-DNB, involving rapid (1-3 days) and widespread exfoliation and degeneration of pachytene spermatocytes and round spermatids, consistent with a direct effect of NB on the Sertoli cell (see Chapter 5).

The experiments described in this chapter have used NB in order to identify novel biochemical markers of early toxicant-induced disruption of spermatogenesis, by evaluating changes in the secretion of specific proteins from isolated ST following exposure to NB. Furthermore, these changes were equated with histological evaluations of testes from rats treated identically in parallel experiments.

6.2. Experimental Procedures

6.2.1. Reagents

Nitrobenzene (NB) was obtained from Sigma (Poole, Dorset, UK). Analysis by gas chromatography/mass spectrometry for composition before use found it to be 99% pure. The vehicle for NB administration was Mazola corn oil (obtained from a supermarket).

6.2.2. Dosing regime

Young adult male rats aged approximately 70 days were administered a single oral dose of NB (300mg/kg bodyweight) by gavage in corn oil (1ml/kg). This dose of NB has been shown previously to cause severe disruption of spermatogenesis *in-vivo* (Bond *et al.*, 1981).

6.2.3. Perfusion-fixation of control and NB-treated rats

At 24 hours after NB-treatment, 2 rats were perfusion fixed according to the methods described in Chapter 3 and data compared to data for 2 perfusion fixed control rats.

6.2.4. Procedure for isolation of ST from control and NB-treated rats

At 12, 24 and 72 hours after NB treatment, seminiferous tubules were isolated from control and NB-treated rats according to the methods described in Chapter 3.

6.2.5. Procedure for isolation of ST from control rats

Seminiferous tubules were isolated from untreated control rats and cultured for 24 or 72 hours in the presence or absence of 10^{-4} M NB according to the methods described in Chapter 3.

6.2.6. Treatment of seminiferous tubule cultures

NB was added to cultures at a dose of 10^{-4} M in dimethyl sulphoxide (DMSO : BDH) at a final concentration of 0.3% (v/v), while controls received an equal volume of this vehicle. Treatments caused no appreciable alteration of the culture medium pH. The 35 S-methionine was added to cultures at the start of incubation and this, together with media and toxicants, was replaced every 24 hours when incubation was for a 72 hour period; media was analysed for protein changes after 24 and 72 hours of culture. The dose of 10^{-4} M NB was chosen as being approximately equivalent to the peak levels which have been achieved *in-vivo* following administration of 300mg/kg NB (Rickert *et al.*, 1983).

6.2.7. Measurement of newly synthesized proteins

Incorporation of 35 S-methionine into both secreted and intracellular proteins was determined by precipitation of aliquots of the culture medium and cell lysates with 10% (v/v) trichloroacetic acid, according to the methods described in Chapter 3.

6.2.8. Two-dimensional SDS PAGE

Newly synthesized 35 S-methionine labelled proteins secreted by isolated ST were evaluated by 2-D SDS PAGE according to the methods described in Chapter 3. ST conditioned medium from control and NB-treated rats from the same experiment were always run in parallel on SDS PAGE, and subsequent determination of any change in the relative abundance of proteins on the gels was by comparison of gels from treated animals with their respective control group from the same experiment. There was little variation between the gels from different experiments.

6.2.9. Collection of plasma and testicular IF

At 24 and 72 hours after NB treatment, blood plasma and testicular interstitial fluid were collected from 6 control and 6 NB-treated rats and stored for measurement of inhibin according to the methods described in Chapter 3.

6.3. Results

6.3.1. Effect of NB on testicular morphology

Within 24 hours of a single oral dose of NB to adult rats, rapid and extensive disruption of spermatogenesis was evident. This consisted of vacuolation and retraction of Sertoli cell cytoplasm, with many pachytene spermatocytes either absent or pyknotic. These changes were stage-specific, with stages VII-XII being affected more than stages I-VI (Figure 6.1).

6.3.2. Effect of NB on testicular weight

Mean testicular weight in rats treated with 300mg/kg NB was significantly lower than in controls at 1 and 3 days post-treatment ($p < 0.01$, Figure 6.2).

6.3.3. Stage-dependent differences in overall protein secretion

The overall level of incorporation of ^{35}S -methionine into proteins secreted into the incubation medium by ST at stages VI-VIII from control rats was more than double that at stages II-V or IX-XII after 24 or 72 hours of culture, as has been reported previously (Sharpe *et al.*, 1992), whereas incorporation of ^{35}S -methionine into intracellular proteins showed no such difference between these stages (Figure 6.3). This difference was evident in the control group in the experiments described below. In view of this and differences between experiments in the level of incorporation of ^{35}S -methionine into ST-secreted proteins, data have been normalized by expressing incorporation as a percentage of the mean control value for that stage.

6.3.4. Effect of NB on overall protein secretion

Following the administration of a single oral dose of NB to rats there was no significant change in the incorporation of ^{35}S -methionine into secreted proteins by ST isolated at stages II-V, VI-VIII or IX-XII of the spermatogenic cycle 12 hours after treatment (Figure 6.4). By 24 hours after NB treatment there was a significant decrease in the incorporation of radiolabel into secreted proteins by ST at stages VI-VIII and IX-XII ($p < 0.001$ and $p < 0.01$ respectively). ST at stages II-V showed no significant change in incorporation when compared with controls (Figure 6.4). With ST isolated from rats 72 hours after the administration of NB, the decrease in incorporation of ^{35}S -methionine into secreted proteins at stages VI-VIII was more pronounced when compared to controls ($p < 0.001$). ST isolated at stages II-V or IX-XII also

Figure 6.1. Representative morphology of seminiferous tubules isolated from control rats at stage VII of the spermatogenic cycle (top) showing pachytene spermatocytes (large arrowheads), round spermatids (small arrowheads) and the heads of elongate spermatids (small arrows) or from rats treated with NB 24 hours earlier (bottom) Sertoli cell nuclei (large arrows), degenerating pachytene spermatocytes (large arrowheads), and normal spermatids (small arrows) and elongate spermatids (small arrows). The interstitial space (S), blood vessels (V) and Leydig cells (L) are shown also . Scale bars = 100 μ m.

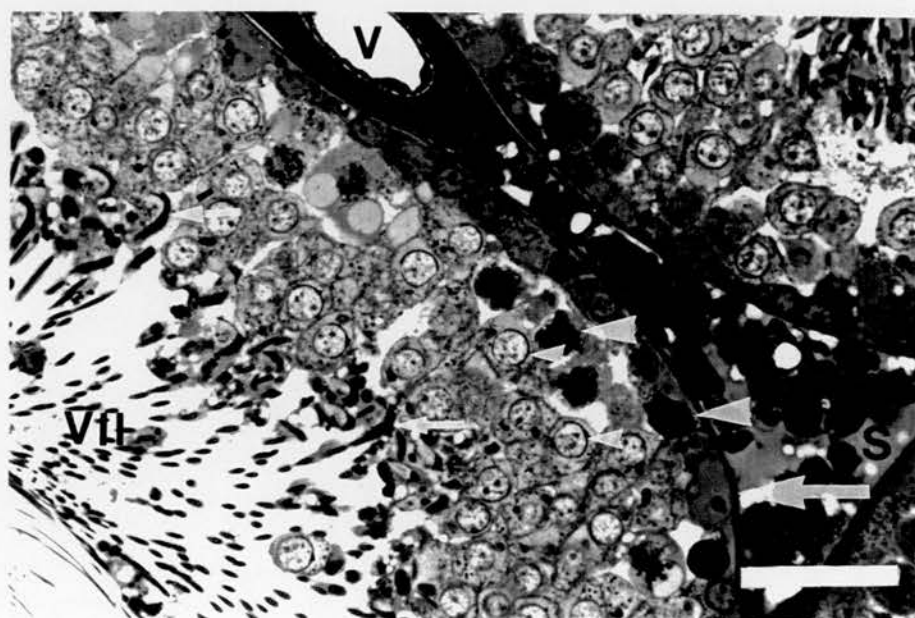
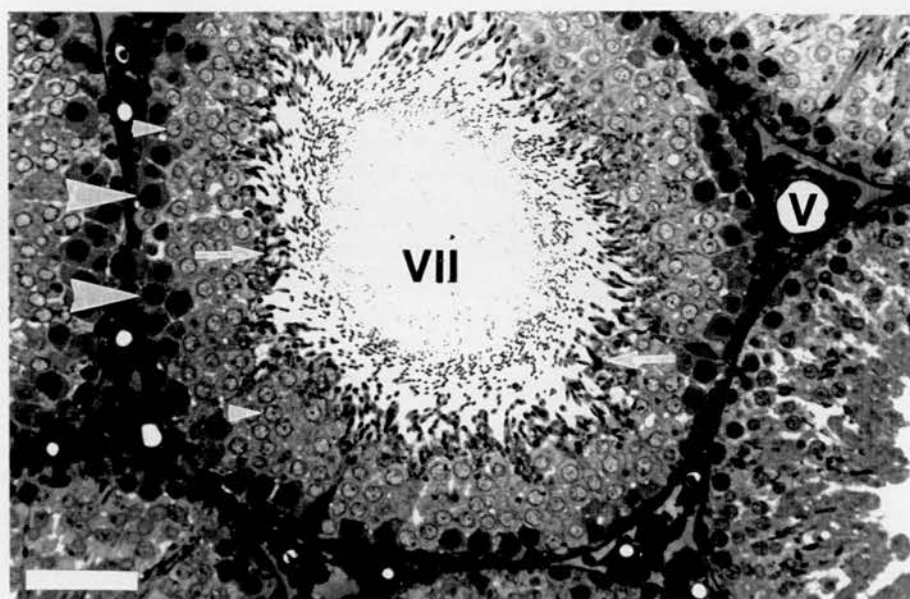


Figure 6.2. Effect of a single oral administration of NB (300mg/kg) (toned bars) or vehicle alone (1ml/kg) (open bars) on testicular weight at 24 or 72 hours after treatment. Values are means \pm SD for 6 rats. ** $p < 0.01$, in comparison to control.

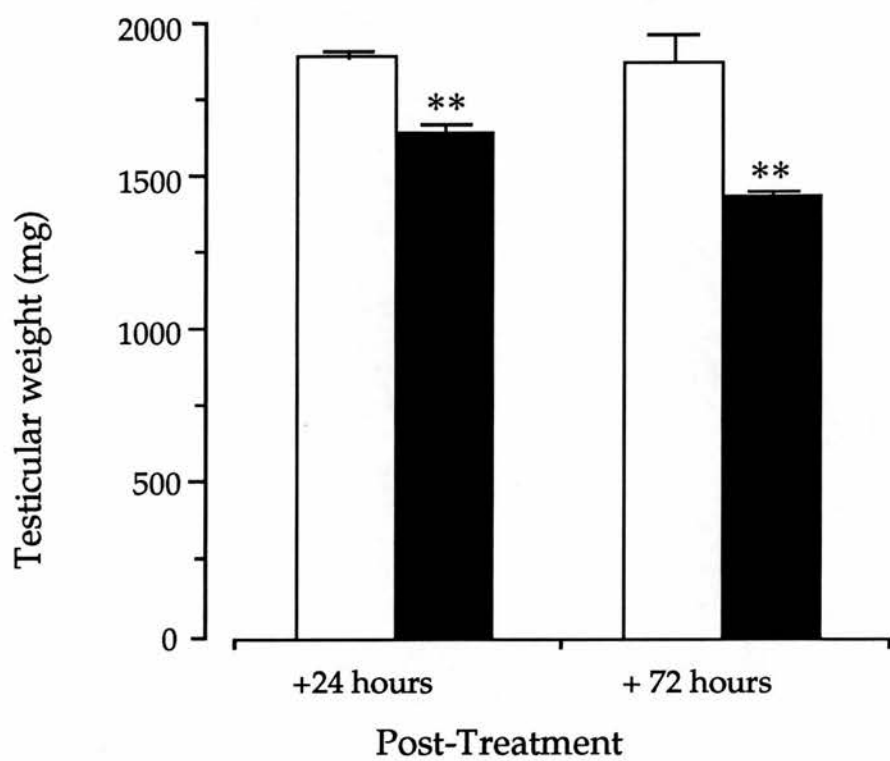
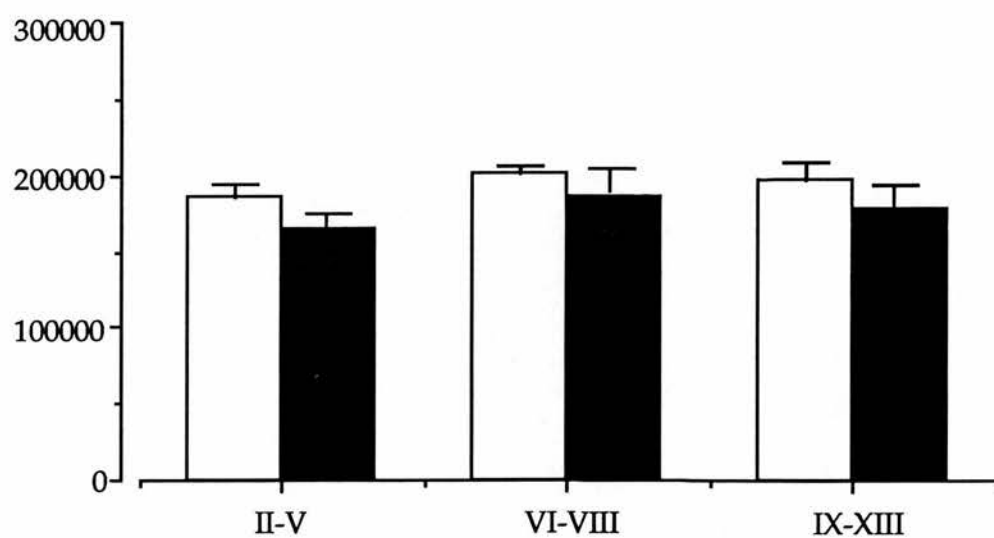
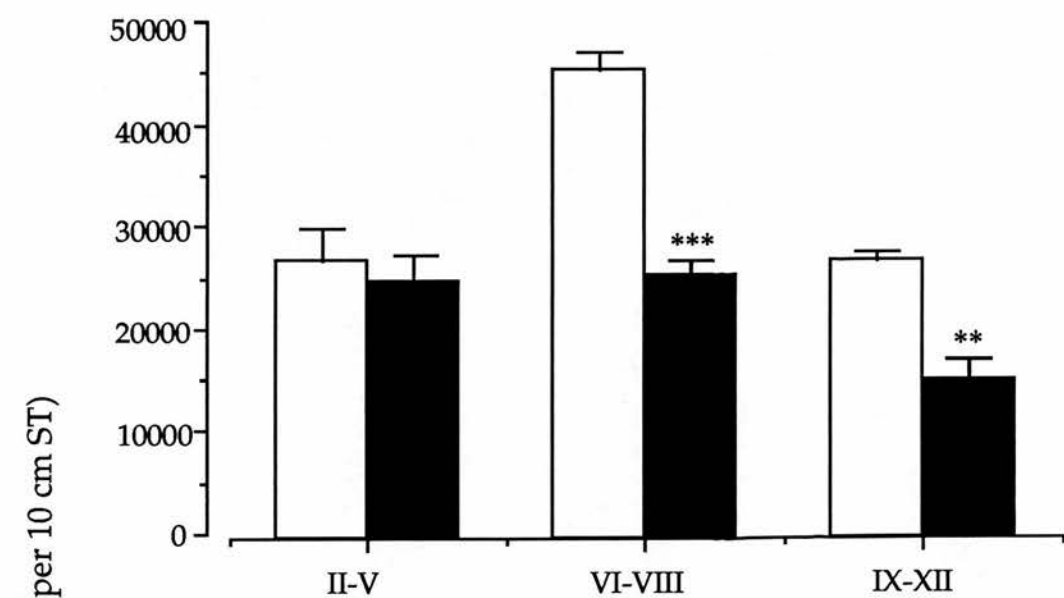
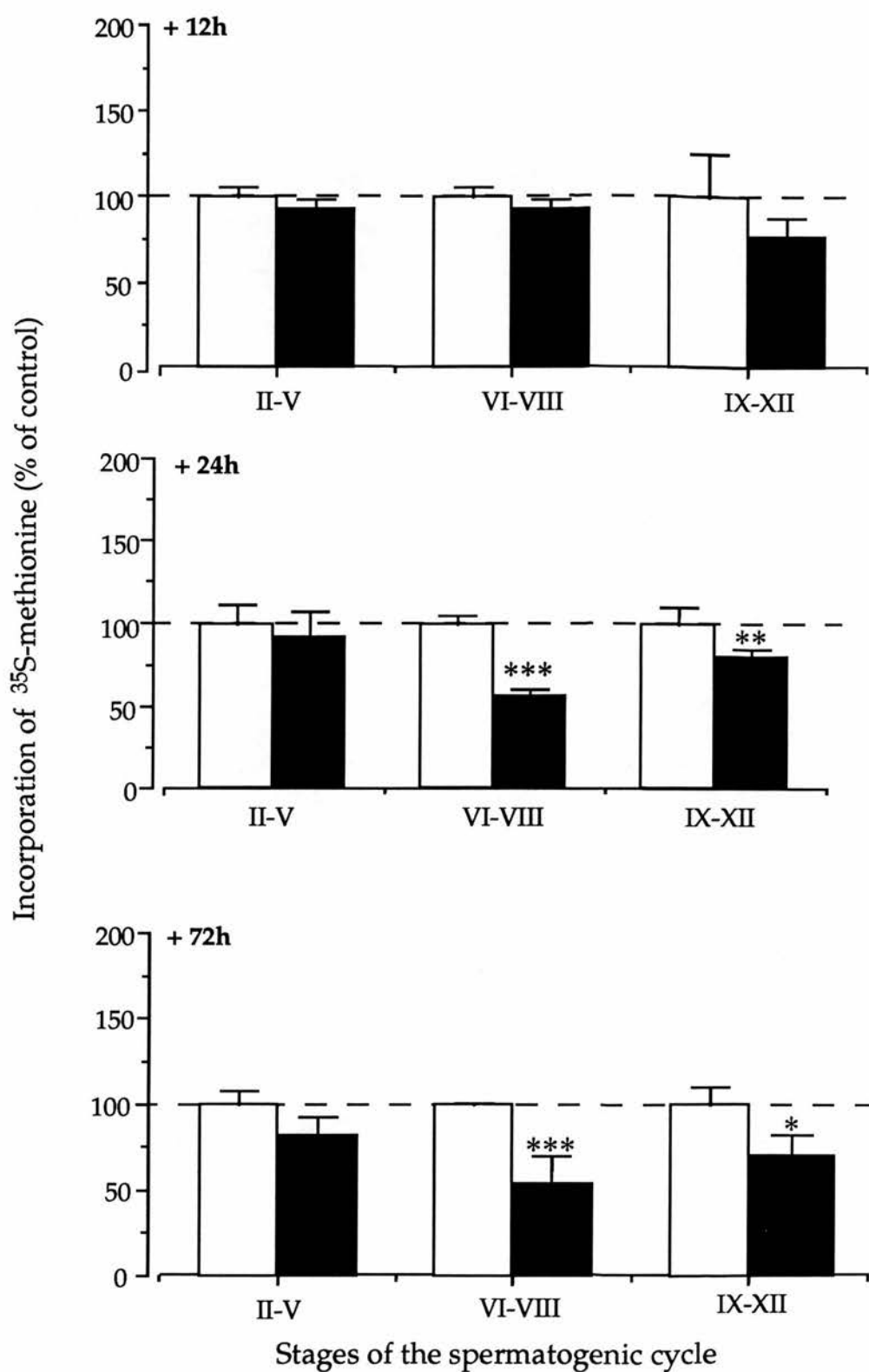


Figure 6.3. Incorporation of ^{35}S -methionine over 24 hours into newly synthesized secreted (top) or intracellular (bottom) proteins by seminiferous tubules isolated at different stages of the spermatogenic cycle from control rats (open bars) or rats pretreated with 300mg/kg NB (toned bars) 24 hours earlier. Values are means \pm SD for three rats per stage group. ** $p < 0.01$, *** $p < 0.001$, in comparison with respective control group.



Stages of the spermatogenic cycle

Figure 6.4. Incorporation of ^{35}S -methionine over 24 hours into newly synthesized proteins secreted *in-vitro* by seminiferous tubules isolated at different stages of the spermatogenic cycle from control rats (open bars) or rats pretreated with 300mg/kg NB (toned bars) either 12 (top), 24 (middle) or 72 (bottom) hours earlier. At each stage incorporation has been expressed as a percentage of the mean control value (dashed line) obtained during the same experiment. Values are means \pm SD of $n=6$. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, in comparison with respective control group.



showed a decrease, although this was slightly less significant ($p < 0.01$ Figure 6.4). A similar picture was obtained with ST that had been isolated from untreated control rats and cultured in the presence of NB (10^{-4}M) for 24 or 72 hours (Figure 6.5). ST at stages VI-VIII and IX-XII which were cultured for 24 hours in the presence of 10^{-4}M NB showed a significant decrease ($p < 0.01$ and $p < 0.05$ respectively) in the incorporation of ^{35}S -methionine into secreted proteins, whereas stages II-V showed no change in incorporation (Figure 6.5). After 72 hours in culture a similar profile of incorporation into secreted proteins was obtained, although the decrease in incorporation at stages IX-XII was not statistically significant (Figure 6.5).

6.3.5. Effect of NB on intracellular proteins

No change in incorporation of ^{35}S -methionine into intracellular proteins at either of the three stage groupings was observed following a single oral dose of NB either 12 or 24 hours earlier (Figure 6.6). This was in marked contrast to the results obtained following a single oral dose of NB 72 hours earlier (Figure 6.6). ST isolated at stages II-V, VI-VIII or IX-XII showed a significant decrease ($p < 0.001$) in incorporation when compared to controls.

6.3.6. Two-dimensional SDS PAGE

The majority of radiolabelled proteins secreted by ST at stages II-V or VI-VIII was unaffected by exposure to NB, but more than fifteen were found to have changed reproducibly. Of these proteins, ten were singled out as being potential markers of NB-induced damage to the testis, on the basis that they showed marked changes in every experiment following exposure to NB. A summary of the molecular weights and isoelectric points of these proteins, obtained from the analyses of six fluorograms, is given in Table 6 and the position of each is circled and labelled on each of the gels.

A comparison of the 2-D profile of proteins secreted by tubules from untreated rats at stages II-V, or VI-VIII (Figures 6.7 and 6.8) revealed that the secretion of several proteins was stage-dependent and occurred predominantly at stages VI-VIII of the spermatogenic cycle (the androgen dependent stages). The secretion of four of the identified potential marker proteins (Numbered 2, 6, 9 and 13) was found to be stage-dependent, occurring predominantly at stages VI-VIII where, following exposure to NB either *in-vivo* or *in-vitro*, they showed reductions in abundance, or were completely absent (Figures 6.7 and 6.8). Four proteins (numbered 1, 4, 7 and

Figure 6.5. Incorporation of ^{35}S -methionine over 24 hours into newly synthesized proteins secreted *in-vitro* by seminiferous tubules isolated at different stages of the spermatogenic cycle from untreated control rats and then cultured in the absence (open bars) or presence (toned bars) of 10^{-4}M NB for either 24 (top) or 72 (bottom) hours. At each stage incorporation has been expressed as a percentage of the mean control value (dashed line) obtained during the same experiment. Values are means \pm SD of $n=4$. ** $p<0.01$, *** $p<0.001$, in comparison with respective control group.

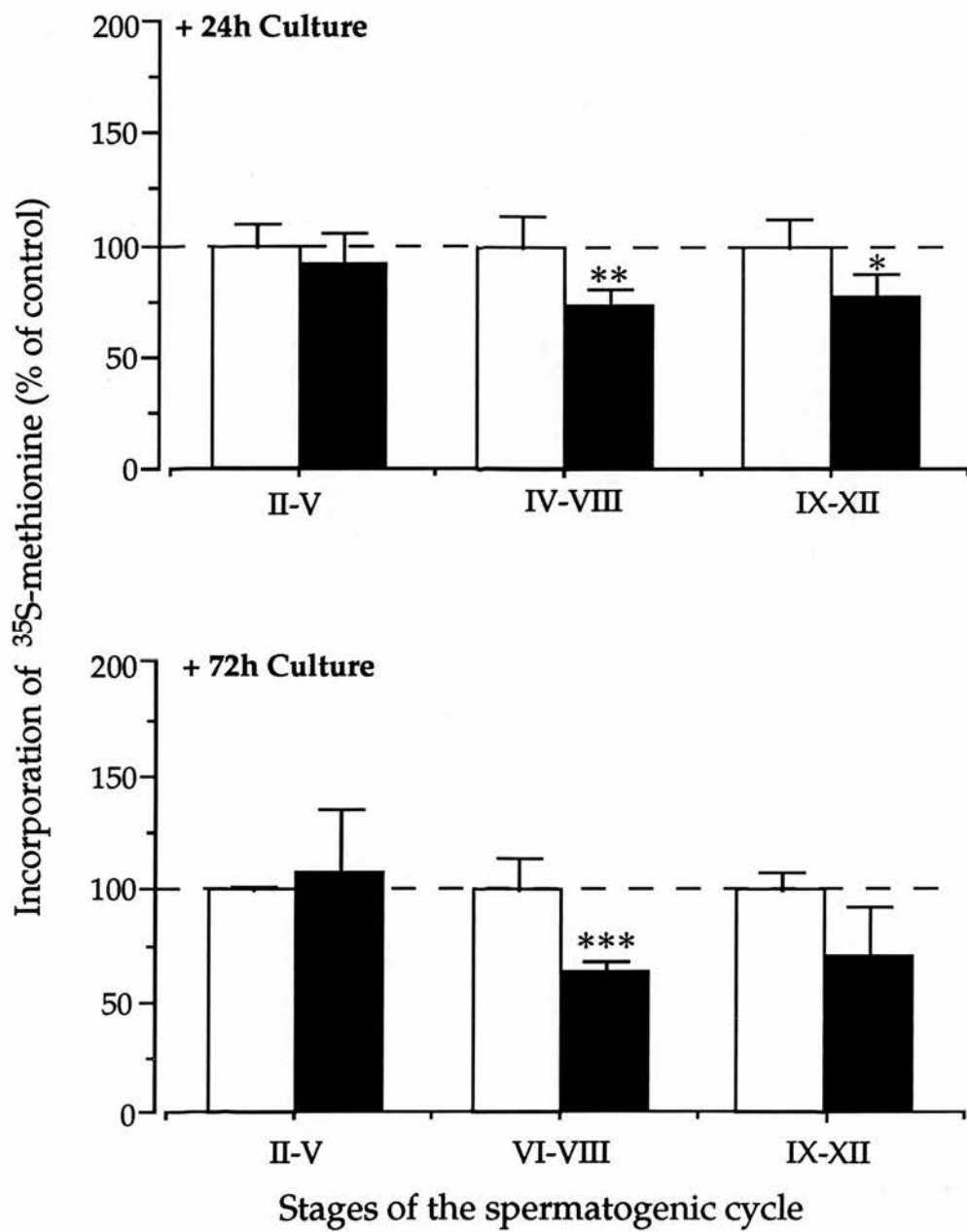
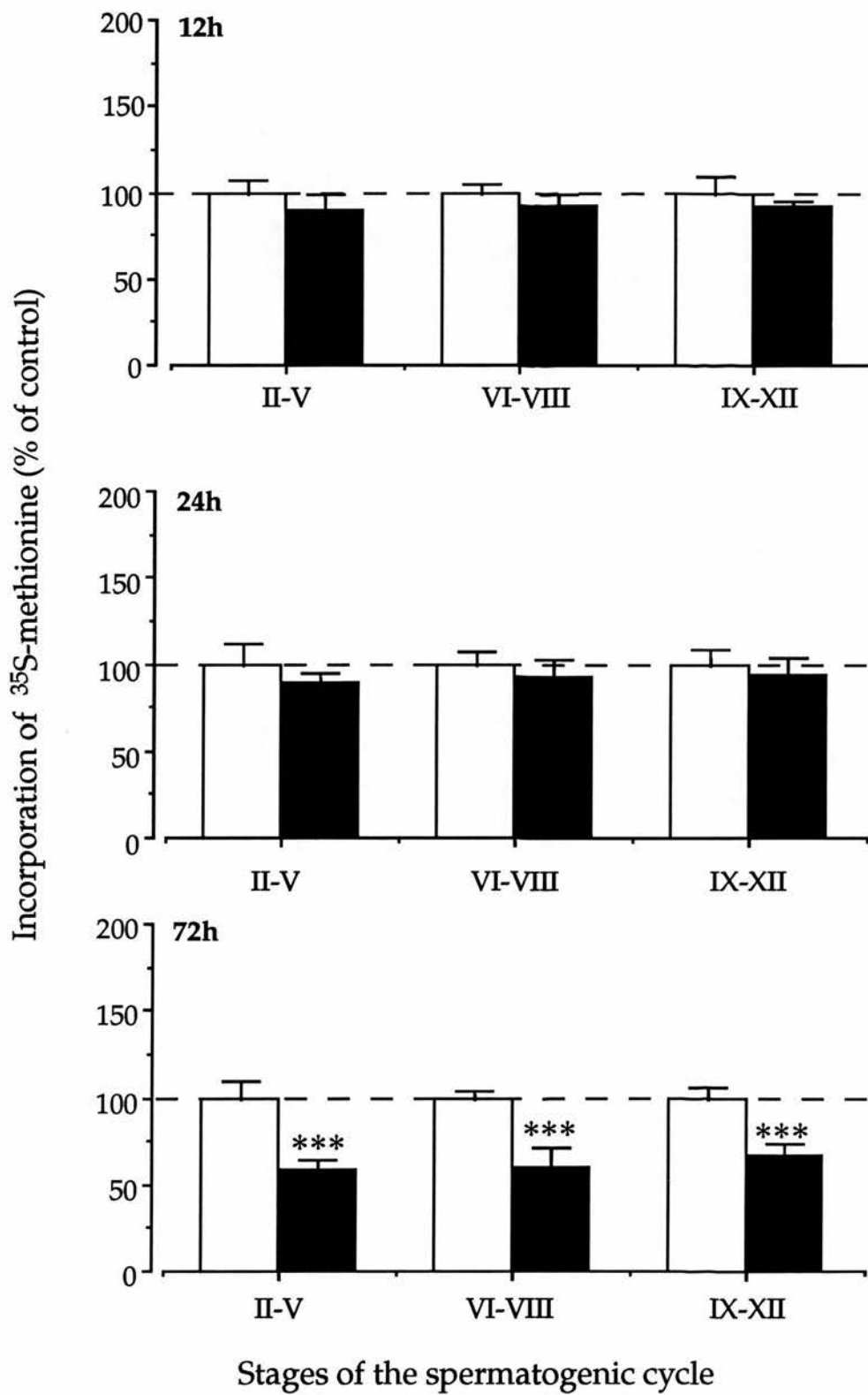


Figure 6.6. Incorporation of ^{35}S -methionine over 24 hours into newly synthesized intracellular proteins by seminiferous tubules isolated at different stages of the spermatogenic cycle from control rats (open bars) or rats pretreated with 300mg/kg NB (toned bars) either 12 (top), 24 (middle) or 72 (bottom) hours earlier. At each stage incorporation has been expressed as a percentage of the mean control value (dashed line) obtained during the same experiment. Values are means \pm SD of n=6. ***p<0.001, in comparison with respective control group.



Protein No	M _r (kDa)	pI	Effect of NB on relative abundance	Stage-dependence of secretion	Possible Identity
1	63 - 73	5.6 - 6.2	Absent or Decrease	All stages	unknown
2	38 - 43	5.7 - 6.2	Decrease	Predominantly at stages VI-VIII	ARP-4 (Sharpe <i>et al.</i> , 1992)
3	28 - 32	5.8 - 6.9	Increase	All stages	Possible charge isomers of SGP-2
4	23 - 27	5.4 - 6.2	Absent or Decrease	All stages	unknown
5	23 - 27	5.8 - 6.3	Induces appearance of protein	All stages	unknown
6	25 - 30	6.9 - 7.4	Decrease	VI-VIII	ARP-3 (Sharpe <i>et al.</i> , 1992)
7	17.5 - 20	5.3 - 6.3	Decrease	All stages	unknown
8	17 - 20	5.9 - 6.4	Decrease	All stages	unknown
9	13 - 15	6.9 - 7.6	Decrease	VI-VIII	ARP-2 (Sharpe <i>et al.</i> , 1992)
13	~9.5	6.9 - 7.4	Decrease	VI-VIII	ARP-1 (Sharpe <i>et al.</i> , 1992)

Table 6. List of seminiferous tubule secreted proteins which showed repeatable differences in relative abundance following treatment with NB (300mg/kg). Protein numbers correspond to those shown in Figures 6.7, 6.8 and 6.9.

Figure 6.7. Incorporation of ^{35}S -methionine into newly synthesized proteins secreted *in-vitro* by seminiferous tubules isolated at stages II-V from control rats (top) or from rats treated 24 hours earlier with NB (300mg/kg) (bottom). Proteins which change in relative abundance following NB exposure are circled and numbered. The positions of the three major Sertoli cell secreted proteins, sulphated glycoprotein-1 (SGP-1), sulphated glycoprotein-2 (SGP-2) and cyclic protein-2 (CP-2), are indicated for reference.

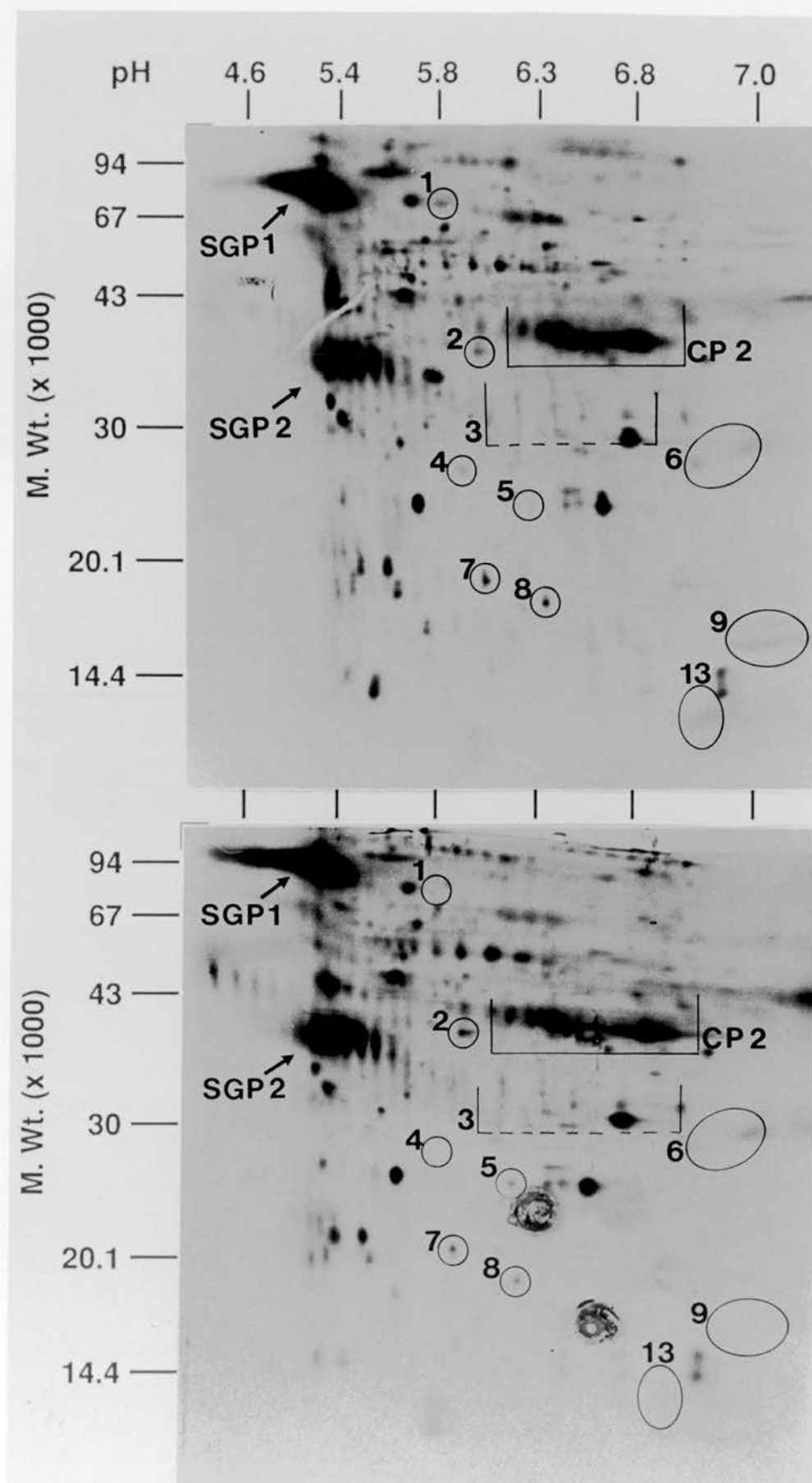


Figure 6.8. Incorporation of ^{35}S -methionine into newly synthesized proteins secreted *in-vitro* by seminiferous tubules isolated at stages VI-VIII from control rats (top) or from rats treated 24 hours earlier with NB (300mg/kg) (bottom). Proteins which change in relative abundance following NB exposure are circled and numbered. The positions of the three major Sertoli cell secreted proteins, sulphated glycoprotein-1 (SGP-1), sulphated glycoprotein-2 (SGP-2) and cyclic protein-2 (CP-2), are indicated for reference.

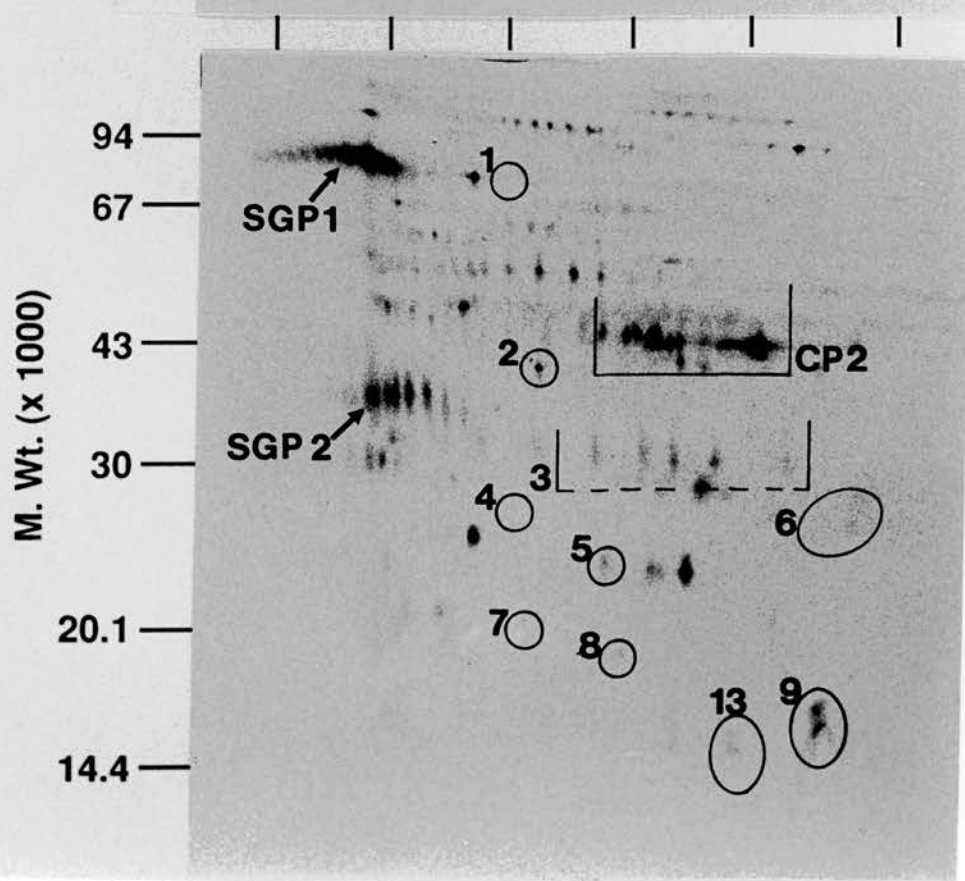
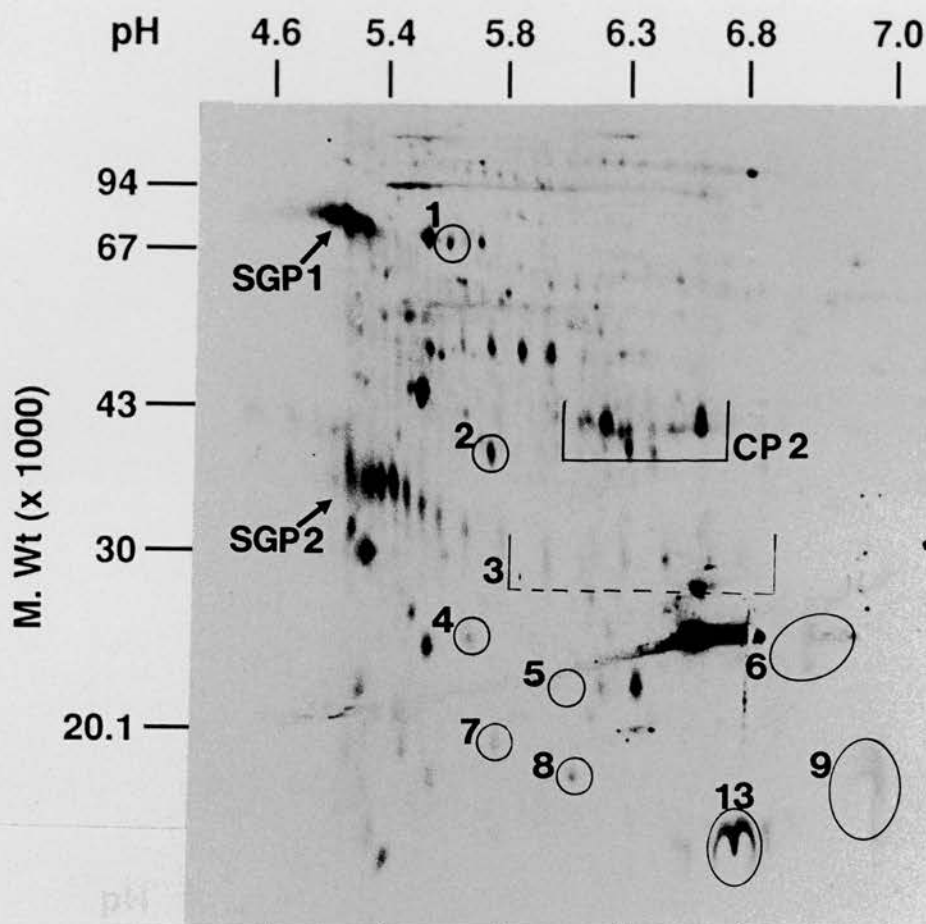
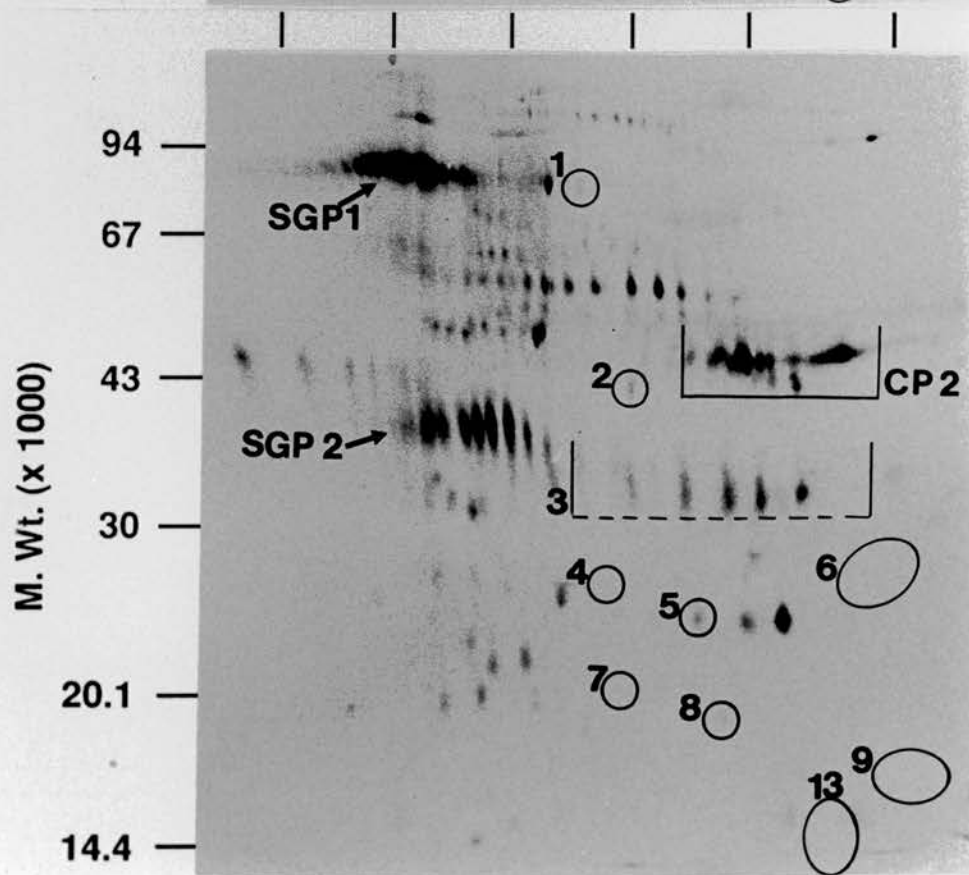
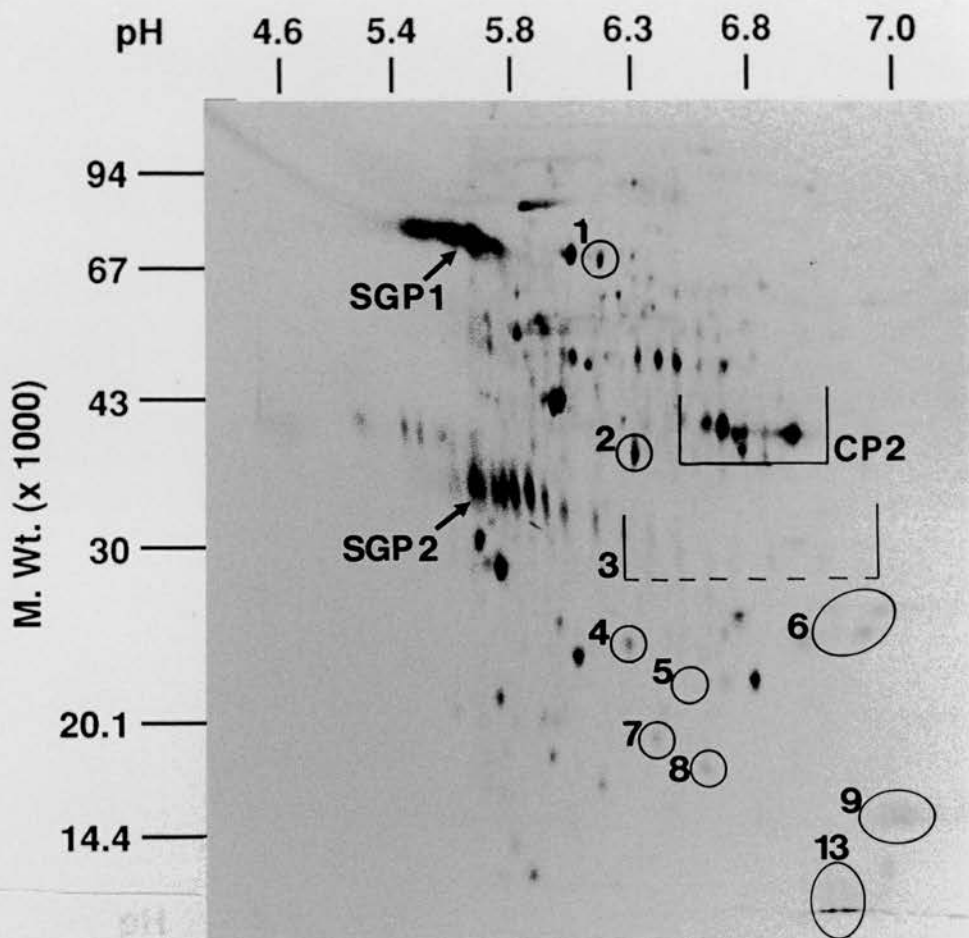


Figure 6.9. Incorporation of ^{35}S -methionine into newly synthesized proteins secreted *in-vitro* by seminiferous tubules isolated at stages VI-VIII from control rats (top) or from rats treated 72 hours earlier with NB (300mg/kg) (bottom). Proteins which change in relative abundance following NB exposure are circled and numbered. The positions of the three major Sertoli cell secreted proteins, sulphated glycoprotein-1 (SGP-1), sulphated glycoprotein-2 (SGP-2) and cyclic protein-2 (CP-2), are indicated for reference.



8) were found to be secreted by ST at stages II-V and VI-VIII and, following exposure to NB they showed pronounced reductions in abundance, or were completely absent. The secretion of protein number 3 was found to increase following exposure to NB, although this was more evident at 3 days after toxicant treatment *in-vivo* (Figure 6.9). The secretion of protein 5 is of particular interest since it would seem that it is either not secreted normally or is secreted in very low amounts by ST from control animals. Following exposure to NB the secretion of protein number 5 was very pronounced at stages VI-VIII and relatively pronounced at stages II-V (Figures 6.7, 6.8 and 6.9).

6.3.7. Effect of NB on immunoactive inhibin secretion

The levels of immunoactive inhibin in IF and plasma were increased significantly at 1 and 3 post-treatment with NB, when compared to the levels in control rats ($p < 0.01$) (Figure 6.10).

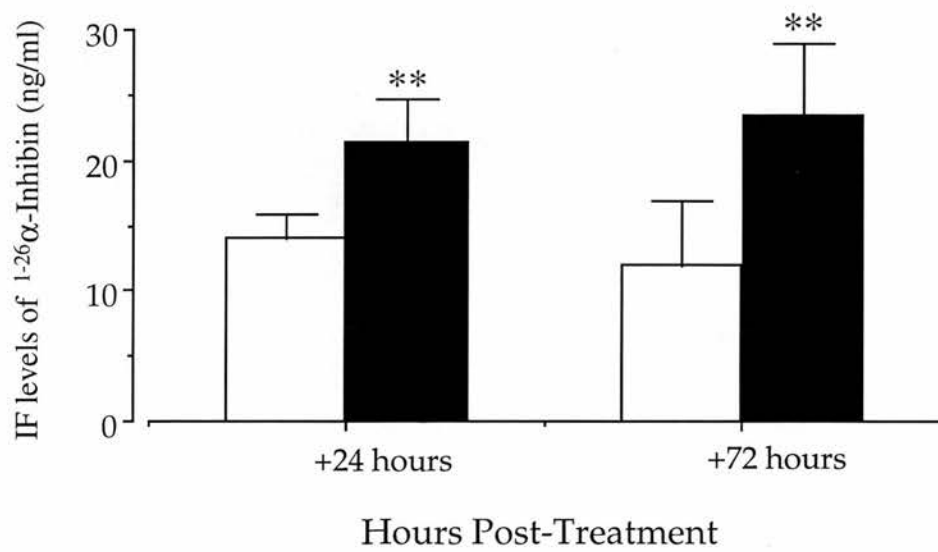
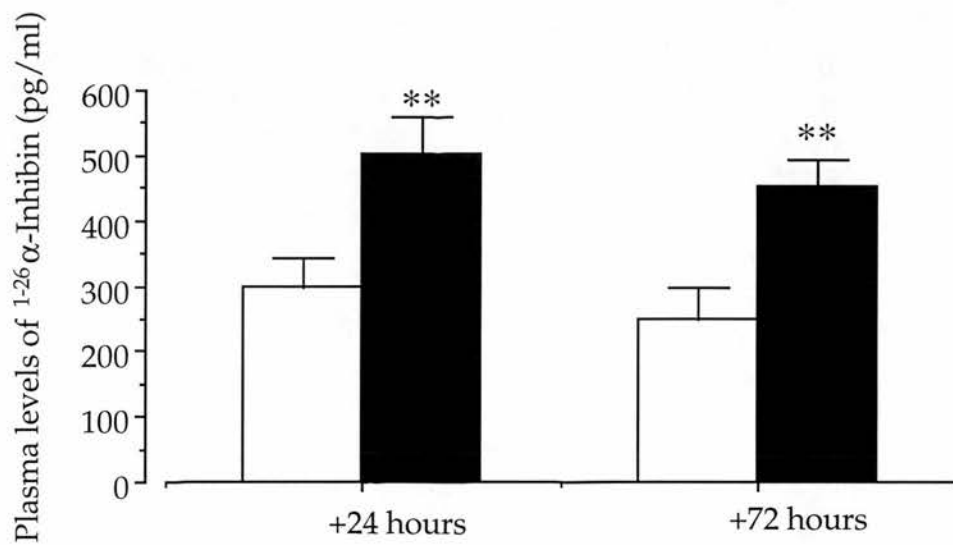
6.4. Discussion

The experiments described in this chapter are the first to evaluate changes in the secretion of proteins by isolated ST following exposure to NB both *in-vivo* and *in-vitro*, and to equate these with histological changes. In addition, testicular weight and the secretion of immunoactive inhibin have been used as indices of testicular dysfunction.

Initial studies concerned with the male reproductive effects of NB were investigated by Bond *et al.* (1981), who observed germ cell necrosis after oral administration of NB to rats. Subsequent studies have established that NB causes testicular damage in rats after acute oral and inhalation administration. These effects have been shown to be dose-dependent and reversible (Medinsky and Irons *et al.*, 1987; Levin *et al.*, 1983; Hamm *et al.*, 1984; Dodd *et al.*, 1987). The toxicity of NB, like that of m-DNB, is considered to be a direct result of perturbation of the Sertoli cell, as demonstrated both *in-vivo* and *in-vitro*, using Sertoli cell cultures in the presence and absence of germ cells (Allenby, 1990; Allenby *et al.*, 1990).

Since NB is very similar structurally to m-DNB, it is presumed that the testicular toxicity observed after treatment with NB is similar to that seen following m-DNB treatment. In the present study, stage-specific changes in

Figure 6.10. Effect of a single oral administration of NB (300mg/kg) (toned bars) or vehicle alone (1ml/kg) (open bars) on levels of immunoactive inhibin in plasma (top) or testicular interstitial fluid (IF) (bottom) collected from rats at 24 or 72 hours after administration. Values are means \pm SD for 6 rats. ** $p < 0.01$, in comparison with respective control group.



incorporation of ^{35}S -methionine into secreted proteins, similar to those observed following exposure to m-DNB (see Chapter 5), were observed following a single oral dose of NB (300mg/kg) to adult rats (Figure 6.4). This dose was chosen because it has been shown to result in serious impairment of spermatogenesis 24 hours after treatment (Bond *et al.*, 1981; Allenby, 1990). When compared with controls, the overall level of incorporation of ^{35}S -methionine into proteins secreted by seminiferous tubules at stages VI-VIII or IX-XII was reduced significantly ($p < 0.001$ and $p < 0.01$, respectively) when isolated 24 hours after treatment (Figure 6.4). There was no significant change in incorporation at stages II-V. By 72 hours after treatment with NB, a pronounced decrease in incorporation of ^{35}S -methionine into secreted proteins occurred with tubules at stages VI-VIII and IX-XII (Figure 6.4). This same profile of incorporation was found in staged tubules that had been isolated from untreated control animals and cultured for 24 or 72 hours in the presence of NB (10^{-4}M), a concentration which Rickert *et al.* (1983) suggested may be achieved following a single oral dose of 300mg/kg (Figure 6.5). In this study, no significant change in the incorporation of ^{35}S -methionine into intracellular proteins was observed until 72 hours post-treatment (Figure 6.6).

The adverse testicular effects of NB *in-vivo*, identified at a histological level in this study, were very similar to those observed for m-DNB, and at 24 hours consisted of vacuolation and retraction of Sertoli cell cytoplasm, coupled with the loss/degeneration of pachytene spermatocytes only at stages VI-XIII. These two initial events were consistent with the published data on the morphological events observed 24 hours after treatment with NB (Bond *et al.*, 1981). The reason for the initial stage-dependent susceptibility of the Sertoli cell and pachytene spermatocytes to the adverse effects of NB and, indeed, m-DNB, is as yet unknown. It is recognized that Sertoli cells exhibit functional changes during the course of the normal spermatogenic cycle (Parvinen, 1982, 1993) and that, during stages VI-XIII of the cycle (the stages initially affected by NB treatment), several key events are initiated within the seminiferous tubule, these being translocation of preleptotene spermatocytes, residual body formation and spermiation, all of which are energy-requiring processes. Whilst in this active metabolic state the Sertoli cells may be particularly vulnerable to toxic insult. As the Sertoli cells are known to play a vital role in the physical and metabolic support of developing germ cells in the seminiferous tubules (Russell, 1980; Jutte *et al.*, 1981; Mita and Hall, 1982; Grootegeod *et al.*, 1984), the loss of such support systems probably results in

the loss of germ cells and, in particular, pachytene spermatocytes which have the highest rate of RNA synthesis of any of the germ cell types (Monesi, 1965) and as such have a high energy demand.

Based on morphological evaluation, the changes observed in the incorporation of ^{35}S -methionine into overall secreted proteins at stages VI-XII at 24 hours after NB treatment accompany germ cell degeneration. The fact that, contrary to the results obtained using m-DNB (see Chapter 5), no change was observed in overall protein secretion by ST isolated 12 hours after treatment with NB, could be due in part to the difference in sensitivity of the Sertoli cell to the adverse effects of m-DNB and NB. m-DNB has been shown previously to disrupt Sertoli cell function *in-vitro*, causing dose-dependent increases in the secretion of lactate (Williams and Foster, 1988), together with dose-dependent exfoliation of viable germ cells (Foster *et al.*, 1987a). In the present study m-DNB, at a single dose level (10^{-4}M), produced increases in the secretion of lactate similar to those reported by previous investigators (Williams and Foster, 1988; Allenby, 1990 and see Chapter 4). However, equimolar doses of NB did not produce equivalent increases in the secretion of lactate or in the degree of exfoliation of germ cells in comparison to m-DNB (see Chapter 4). This suggests that m-DNB is more toxic to the Sertoli cell than NB, and it is this which probably accounts for the 6-fold difference in the dose of NB (300mg/kg) required to produce a similar degree of testicular disruption following a single oral dose of m-DNB (50mg/kg).

Analysis of ST-secreted proteins by 2-D SDS PAGE identified ten proteins which showed major reproducible changes in secretion following exposure to NB. In most cases (proteins numbered 1, 2, 4, 6, 7, 8, 9 and 13 in Figures 6.7 and 6.8) their secretion was reduced markedly. A comparison of proteins secreted by tubules from untreated animals at stages II-V or VI-VIII revealed that four of the proteins identified are stage-dependent (2, 6, 9 and 13), being secreted almost exclusively at stages VI-VIII, which are known to be androgen-dependent (Sharpe *et al.*, 1992). The identity of these four proteins has yet to be established, but they correspond in molecular weight and pI to four recently identified androgen-regulated and germ cell-dependent proteins, namely, ARP 1, 2, 3 and 4 (Sharpe *et al.*, 1992; McKinnell and Sharpe, 1992). Exposure of ST to NB resulted in the secretion of a protein with an apparent molecular weight of 26 kilodaltons (numbered 5 in Figures 6.7, 6.8 and 6.9). This protein was not secreted by ST isolated from control adult animals, but was shown to be secreted by ST isolated from control immature animals (see Chapter 4). The secretion of protein 3 was found to

increase following exposure to NB; this was most evident at 72 hours after exposure, particularly at stages VI-VIII of the spermatogenic cycle (Figure 6.9). Similarly, increased secretion of this protein was found following exposure to m-DNB (see Chapter 5).

The cellular source of the ten identified proteins remains to be established. From the present studies it is not possible to say whether these proteins derive from Sertoli, peritubular or germ cells. However, a recent study by McKinnell and Sharpe, (1992) has shown that the secretion of protein number 6 is decreased when pachytene spermatocytes are depleted selectively from the seminiferous tubule, whilst proteins numbered 4 and 9 are absent or decreased markedly when round spermatids are missing from the tubule. These results could mean either that protein 6 and proteins 4 and 9 are secretory products of pachytene spermatocytes and round spermatids respectively, or that they are products of the Sertoli cells, the secretion of which is absolutely dependent upon the presence of pachytene spermatocytes or round spermatids. The fact that protein changes were observed which either precede (stages II-V) or accompany (stages VI-VIII) germ cell degeneration suggests that, in the first instance, the secretory function of the Sertoli cell has been perturbed in such a way that it no longer provides biochemical and structural support for developing germ cells. It follows therefore that when one or more germ cell types are missing, secondary changes in Sertoli cell secretory function may occur, and such changes may influence further the development of the germ cells that still remain in contact with the Sertoli cell.

The present study has also measured an index of Sertoli cell secretory function, namely inhibin secretion. Following a single oral dose of NB, levels of immunoactive inhibin in plasma and testicular IF were increased significantly ($p < 0.01$) at 1 and 3 days post-treatment (Figure 6.10), coincident with the depletion of pachytene spermatocytes from the seminiferous tubule. Similarly, blood levels of FSH have been shown previously to follow a similar pattern of change (Allenby, 1990). Raised serum levels of FSH are widely considered to reflect major disruption of normal testicular function and germ cell degeneration *in-vivo* (de Kretser and Kerr, 1983). Furthermore, previous studies have shown that, in several situations in which spermatogenesis is impaired, inhibin levels in IF increase proportionately in the presence of increased levels of serum FSH (Sharpe *et al.*, 1988; Sharpe and Maddocks, 1989; Maddocks and Sharpe, 1989; Allenby *et al.*, 1991a). It is not possible to make a definite conclusion as to the cause of the increase in the secretion of

immunoactive inhibin 1-3 days after NB treatment, but this increase could be due to a direct stimulatory effect of NB on the Sertoli cell or to the depletion of pachytene spermatocytes having an indirect effect on Sertoli cell function. The direct stimulatory effect of NB on the secretion of immunoactive inhibin secretion is of interest since this compound, like m-DNB, has been shown previously to induce an increase in the secretion of lactate and pyruvate by Sertoli cells in culture, an effect which is specific to testicular toxicants (Williams and Foster, 1988; Allenby *et al.*, 1991a; see Chapter 4).

In conclusion, the experiments presented in this chapter have shown that NB exposure causes stage-specific changes in the secretion of proteins by isolated ST. Analysis by 2-D SDS PAGE has enabled the identification of ten potential markers of testicular damage induced by NB, and it is presumed that changes in these proteins may mediate some of the adverse testicular effects of NB. All of these potential marker proteins were found to change following exposure to the chemically-related Sertoli cell toxicant m-DNB (see Chapter 5). Taken together, results such as these suggest that some, if not all, of these proteins may be specific markers of Sertoli cell dysfunction.

7. Effect of MAA on ST protein secretion

Chapters 5 and 6 identified early stage-specific changes in the secretion of both overall and specific proteins by isolated ST following exposure to two well known Sertoli cell toxicants, namely, meta-dinitrobenzene and nitrobenzene. In this chapter the effects of selective depletion of pachytene spermatocytes from the seminiferous tubule by the germ cell specific toxicant methoxyacetic acid were examined.

7.1. Introduction

The glycol ethers are a family of solvents which have found broad use industrially, due to their ready solubility in water and many organic solvents. The National Institute of Occupational Safety and Health (U.S.A.) estimates that between 200,000 and 2,000,000 workers are exposed to each of six glycols, glycol ethers or glycol ether acetates (reviewed by Hardin, 1983). As a result of their unique solvent characteristics, glycol ethers in general have found numerous applications as, for example, resin solvents in surface coatings and inks, hydraulic brake fluids, and dye solvents in textile and leather applications.

Some of the economically important glycol ethers used in the chemical industry are the low molecular weight ethylene glycol mono-n-alkyl ethers, examples of which are ethyl glycol monomethyl ether (EGME), ethyl glycol monoethyl ether (EGEE) and ethylene glycol monobutyl ether (EGBE). These compounds are also known under their synonyms of 2-methoxyethanol, 2-ethoxyethanol and 2-butoxyethanol, respectively.

The glycol ethers are readily absorbed, either percutaneously, or by inhalation or ingestion. It has been known for some time now that EGME, in particular, can produce haematological disorders in both humans (Donley, 1936; Greenburg *et al.*, 1938; Parsons and Parsons, 1938) and laboratory animals (Werner *et al.*, 1943a,b,c). In both humans and animals sufficient exposure can occur through contact with the skin to produce toxicity (Rowe and Wolfe, 1982). High doses may even cause death either by respiratory tract arrest or renal failure.

That glycol ethers can produce testicular damage has been known for some time. Wiley *et al.* (1936), for example, described testicular disruption induced by inhalation of EGME in rats and mice. The same group, in 1938, reported similar effects in rabbits after treatment with EGME. The results of a study by Morris *et al.* (1942), were however, contradictory in that they showed

no effect in rabbits and rats. Because of this discrepancy the potential of glycol ethers to induce testicular toxicity was not appreciated fully until more recently, when Nagano *et al.* (1979) investigated the testicular effects of a range of ethylene glycol mono-n-alkyl ethers in mice. These authors found that EGME, EGME acetate, and EGEE were the most potent in their effects on the testis. The earliest effects on relative testis weight with EGME occurred at the 250mg/kg/day dose level after 5 weeks of oral administration. On the basis of their results, Nagano *et al.* (1979) were able to determine a ranking order for the production of testicular damage in these species to be EGME (and its acetates) >EGEE (and its acetates) >EGBE. Subsequent studies have shown that glycol ethers produce testicular toxicity in a number of species including the rat, mouse, dog, rabbit and chicken, a species variation wider than most testicular toxicants (Hardin, 1983). A recent comparative study (Miller *et al.*, 1981) has shown EGME to be capable of producing a decrease in testicular weight following a 2 week inhalation exposure of the compound at a dosage of 100ppm in the rat and mouse. A subsequent inhalation study by Miller *et al.* (1983) revealed that exposure to EGME at concentrations up to 100ppm for periods from 9 days to 13 weeks produced testicular changes as well as adverse effects on bone marrow and lymphoid tissues in rats, mice and rabbits. The changes in the seminiferous epithelium of rats after 9 days of inhalation exposure to 100ppm were diffuse and severe, with degeneration and necrosis of germ cells in addition to the formation of giant bodies. Foster *et al.* (1983) have shown the effects of both EGME and EGEE via the oral route of administration to rats, with EGME proving again to be more active than EGEE.

The initial cell site of damage following an oral administration of either EGME and EGEE to rats was investigated by Foster *et al.* in 1983. These authors identified the pachytene and secondary spermatocyte as the initial testicular target for the effects of EGME and EGEE within 24 hours after a single oral dose of 100mg/kg. In addition, at 16 hours after a single oral dose of 500mg/kg, they observed mitochondrial swelling and disruption of spermatocyte, cytoplasmic vacuolation and premature condensation of nuclear chromatin (Foster *et al.*, 1983). Similarly, EGME has been shown to affect primary and secondary spermatocytes within 24 hours of exposure to 100ppm, with some vacuolation of Sertoli cells also occurring (Samuels, 1983). Whilst Doe *et al.* (1983) also observed degeneration of primary spermatocytes and spermatids, with spermatogonia, Sertoli and Leydig cells apparently unaffected in rats exposed to 300ppm EGME for 10 days.

Quantitative studies have demonstrated further that certain stages in the meiotic development of spermatocytes are more susceptible to the toxicity of EGME and EGEE (Chapin *et al.*, 1984; Creasy *et al.*, 1984), these being early pachytene (stages I, II and V) or late pachytene (stages XI-XIV) (Creasy *et al.*, 1985). Studies by Chapin *et al.* (1985) described the morphological changes in the reproductive system of male rats in order to identify the morphogenesis of the lesion and to correlate further histological changes with androgen-binding protein (ABP) activity found in fluid collected from the rete testis following efferent duct ligation. Using Fischer-344 rats that had received an oral dose of 150mg/kg/day EGME for 5 days/week, Chapin and co-workers (1985) detected necrotic changes in meiotic spermatocytes 24 hours after a single dose. These effects were more pronounced following subsequent doses. Pronounced maturation depletion of germ cells was apparent at later time intervals and was manifest as an absence of round spermatids from tubules in stages I and III. These effects continued such that, by 7 to 10 days post-treatment, only Sertoli cells, spermatogonia and late spermatids were present in the seminiferous epithelium. No changes in the relative amounts of ABP collected from the rete testis were, however, detected. The authors concluded that the degree of testicular lesion induced by EGME was dependent upon the dose and frequency of administration, with lower single doses affecting only a few specific germ cell types (pachytene spermatocytes) at particular stages of the spermatogenic cycle, whilst higher single and multiple doses affected a larger population of pachytene spermatocytes within a number of stages.

Recent metabolic studies have shown that the observed testicular effects of glycol ethers are not due to the parent compound, but are due to specific metabolites produced *in-vivo* from the parent compound. These studies have shown that the glycol ethers are converted to the corresponding alkoxyacetic acids (which in some instances may be conjugated to glycine) (Hutson *et al.*, 1979; Jönsson *et al.*, 1978, 1982; Miller *et al.*, 1983; Moss *et al.*, 1985), a process mediated by alcohol dehydrogenase, presumably via the alkoxyacetaldehyde intermediate (Jönsson *et al.*, 1982; Miller *et al.*, 1983; Moss *et al.*, 1985). This conversion results in the formation of methoxyacetic acid (MAA) and ethoxyacetic acid (EAA) from EGME and EGEE metabolism respectively. Foster *et al.* (1983) reported that treatment with methoxyacetic acid produced testicular lesions identical to those seen after treatment with EGME, and with methoxyaldehyde, the postulated intermediate in the conversion of MAA, as demonstrated by the urinary profile and the plasma

metabolite concentrations. At the same time pyrazole, (an alcohol dehydrogenase inhibitor), was found to give complete protection against the testicular toxicity of EGME, thereby demonstrating the importance of the metabolic activation of EGME in producing testicular toxicity (Moss *et al.*, 1985). The *in-vitro* results described by Gray *et al.* (1985) give further support for the identification of MAA as the ultimate factor responsible for the adverse testicular effects of EGME. Recent studies by Beattie *et al.* (1984) demonstrated that exposure of Sertoli cells in culture to MAA, but not to EGME itself, decreased the production of lactate but had no significant effect on total protein synthesis as measured by [^3H] leucine incorporation. Exposure to EGME *in-vivo* was also found to decrease testicular lactate levels (Beattie *et al.*, 1985). It was suggested by these authors that a decrease in the levels of lactate may have detrimental effects on the viability of spermatocytes, which are the primary target cell following EGME exposure *in-vivo*. These observations are significant since Jutte *et al.* (1981) reported that maximum stimulation of oxygen consumption and RNA and protein synthesis by pachytene spermatocytes or round spermatid preparations required an exogenous source of lactate. Degeneration of these cells was readily apparent within 24 hours after incubation without lactate. Jutte *et al.* (1982) found that isolated spermatocytes did not survive in the presence of glucose. It was therefore suggested by Beattie *et al.* (1984, 1985) that inhibition of lactate production *in-vitro* and possibly *in-vivo* resulted in pachytene spermatocyte degeneration. However, Gray *et al.* (1985) found that the exogenous administration of lactate to mixed germ cell Sertoli cell+germ cell co-cultures did not protect against MAA-induced pachytene spermatocyte degeneration, suggesting that the lack of endogenously produced lactate was not the cause of pachytene spermatocyte necrosis.

EAA has also been shown to produce similar testicular damage to that seen with the corresponding parent glycol ether after repeated high dose, oral administration (Foster *et al.*, 1983; Creasy and Foster, 1984). This contrasts with the results of studies by Gray *et al.* (1985), who reported that the repeated administration of high doses (868mg/kg/day for 4 days) to immature (aged 31 day old) rats had no effect on testicular weight or histology. Foster *et al.* (1987b) provided evidence to suggest that the testicular toxicity of alkoxyacetic acid diminishes with increasing chain length. They demonstrated *in-vivo* that pachytene spermatocytes in stages XIII-XIV and I were the initial primary site of toxicity for MAA and EAA within 24 hours of treatment, whilst n-butoxyacetic acid (BAA) had no discernible effect on the

testis at any dose level or time. Similarly, the addition of MAA, EAA and BAA to testicular cell cultures at concentrations approximately equivalent to the steady state plasma levels of MAA achieved after a testicular toxic dose (500mg/kg) of EGME caused a specific loss of pachytene spermatocytes (the known target cell type *in-vivo*) from the system, causing a greater loss than EAA. BAA was without any effect in testicular cell populations *in-vitro*. Thus, a strong correlation exists between the effects of MAA *in-vivo* and *in-vitro*.

The administration of MAA to rats *in-vivo* has been reported to cause the selective and stage-specific destruction of pachytene spermatocytes. A dose of 650mg/kg results in the degeneration of pachytene spermatocytes at all stages of the spermatogenic cycle except for pachytene spermatocytes at early- to mid-stage VII (Foster *et al.*, 1983, 1984, 1987; Creasy *et al.*, 1985; Bartlett *et al.*, 1988). The mechanism by which MAA exerts this selective effect is unknown, as is the reason why a particular subset of pachytene spermatocytes at early- to mid-stage VII is resistant to the effects of MAA. Recently Saunders *et al.* (1993) have suggested that the high levels of expression of Cox II mRNA, which codes for the terminal enzyme in the electron transport chain located in mitochondria in pachytene spermatocytes at these same stages, may explain why these cells are less susceptible to the adverse effects of MAA.

MAA has been used as an experimental tool with which to deplete the seminiferous epithelium of specific germ cell types using maturation depletion. Recently, Ratnasooriya and Sharpe (1989) found that exposure to high doses of MAA (650mg/kg), in addition to causing the specific loss of pachytene and later spermatocytes at all stages other than early- to mid-stage VII, resulted in a number of unexpected changes at 21 days post-treatment. These were relatively minor but included a reduction in the number of pachytene spermatocytes at late stage VII/early stage VIII, a retention of sperm at stages IX-XIV and an increased degeneration of pachytene spermatocytes and round spermatids at stage VII and of secondary spermatocytes at XIV-I. Taken together, results such as these suggest that some disturbance of normal paracrine inter-relationships had occurred 21 days after MAA treatment. The authors concluded, however, that, whilst these deleterious changes may occur secondarily following MAA treatment, spermatogenesis proceeds for the most part normally and fertility is largely maintained despite a massive but transient decrease in motile sperm output.

Recovery studies, suggest that high doses of EGME cause reversible damage (Foster *et al.*, 1983; Rao *et al.*, 1983; Ondiz *et al.*, 1984).

Studies on paracrine interactions in the testis have concentrated on work with isolated cells or co-cultures of mixed cell types. However, as the role of paracrine interactions is to co-ordinate the functions of all testicular cell types (Sharpe, 1986) observations from cell cultures *in-vitro* may have limited physiological relevance. An alternative approach has therefore been to use MAA as a tool with which to study paracrine interactions *in-vivo*. Bartlett *et al.* (1988), using MAA, studied the effects of germ cell depletion *in-vivo* on the pituitary-testicular endocrine axis. Following a single oral dose of 650mg/kg of MAA, hormonal measurements were evaluated, and histological observations were made at specific time points after treatment. The results showed that the levels of FSH in serum were increased at 1 and 3 days after treatment. FSH levels then returned to control values before reaching a second peak at 21 days after MAA treatment. Levels of ABP in interstitial fluid exhibited an identical pattern. In contrast, the levels of LH and testosterone were unaffected by treatment. The authors suggested that this biphasic response to MAA was related to the germ cell type missing from the seminiferous epithelium from the majority of tubules after treatment. Similarly, Allenby *et al.* (1991a) showed that the secretion of immunoactive inhibin by Sertoli cells was modulated by the depletion of elongate spermatids (and possibly pachytene spermatocytes), whilst the depletion of other germ cell types was without an effect on the secretion of inhibin.

The experiments described in this chapter used MAA in order to identify novel biochemical markers of toxicant-induced damage to spermatogenesis, by evaluating changes in the secretion of specific proteins from isolated ST following exposure to MAA. MAA was used in these studies because it is a testicular toxicant which is thought to act on pachytene spermatocytes, unlike m-DNB and NB which are known to act on Sertoli cells, but which causes the loss/degeneration of pachytene spermatocytes, thereby providing a useful comparison.

7.2. Experimental Procedures

7.2.1. Reagents

Methoxyacetic acid was obtained from Aldrich Chemical Co. Analysis by gas chromatography/mass spectrometry for composition before use found it to

be >99% pure. To prepare a stock solution of MAA, 10.8ml (pH 0.5) was diluted with concentrated sodium hydroxide to pH 7.4. The solution was then made up to 45mls with 0.9% (w/v) saline.

7.2.2. Dosing Regime

Young adult male rats aged approximately 70 days were administered a single oral dose of MAA (650mg/kg/bodyweight) by gavage. Control animals received an equivalent volume of saline vehicle. This dose of MAA has been shown previously to cause major depletion or loss of pachytene and later spermatocytes at all stages of the spermatogenic cycle other than early- to mid-stage VII, within 24 hours of administration (Bartlett *et al.*, 1988).

7.2.3. Procedure for isolation of ST from control and MAA-treated rats

At 24 and 72 hours after MAA treatment, seminiferous tubules were isolated from control and treated rats and cultured according to the methods described in Chapter 3.

7.2.4. Procedure for isolation of ST from control rats

Seminiferous tubules were isolated from untreated control rats and cultured for 24 or 72 hours in the presence or absence of 10^{-4} M MAA according to the methods described in Chapter 3.

7.2.5. Treatment of ST cultures

MAA was added to cultures at a dose of 10^{-4} M in 0.9% (w/v) saline, corrected to pH 7.4, while controls received an equal volume of this vehicle. Treatments caused no appreciable alteration of the culture medium pH. The 35 S-methionine was added to cultures at the start of incubation and this, together with the media and toxicants, was replaced every 24 hours when incubation was for a 72 hour period; media was analysed for protein changes after 24 and 72 hours of culture. The concentration of MAA used in culture was 15-fold less than peak blood levels following a single oral dose of 650mg/kg (P.M.D. Foster - personal communication).

7.2.6. Measurement of newly synthesized proteins

Incorporation of 35 S-methionine into secreted proteins was determined by precipitation of aliquots of the culture medium with 10% (v/v) trichloroacetic acid, according to the methods described in Chapter 3.

7.2.7. Two-dimensional SDS PAGE

Newly synthesized ^{35}S -methionine labelled proteins secreted by isolated ST were evaluated by 2-D SDS PAGE according to the methods described in Chapter 3. ST conditioned medium from control and MAA-treated rats from the same experiment were always run in parallel on SDS PAGE, and subsequent determination of any change in the relative abundance of proteins on the gels was by comparison of gels from treated animals with their respective control group from the same experiment. There was little variation between the gels from different experiments.

7.2.8. Collection of plasma samples and testicular IF

At 24 and 72 hours post-treatment, plasma and testicular interstitial fluid were collected from 5 control and 5 MAA treated rats and stored for the measurement of immunoactive inhibin using the methods described in Chapter 3.

7.3. Results

7.3.1. Effect of MAA on testicular weight

Mean testicular weight in rats treated with 650mg/kg MAA was reduced significantly at 3 days ($p < 0.01$) but not at 1 day post-treatment (Figure 7.1).

7.3.2. Stage-dependent differences in overall protein secretion

The overall level of incorporation of ^{35}S -methionine into proteins secreted into the incubation medium by ST at stages VI-VIII from control rats was more than double that at stages II-V or IX-XII (Figure 7.2). In view of this and differences between experiments in the actual level of incorporation of ^{35}S -methionine into ST-secreted proteins, data have been normalized by expressing incorporation as a percentage of the mean control value for that stage. It is emphasized, however, that, within each experiment differences comparable to those shown in Figure 7.2 were observed.

7.3.3. Effect of MAA on overall secreted proteins

Following the administration of a single oral dose of MAA to rats there was a significant decrease ($p < 0.01$) in the incorporation of ^{35}S -methionine into

Figure 7.1. Effect of a single oral administration of MAA (650mg/kg) (toned bars) or vehicle alone (2.9mg/kg) (open bars) on testicular weight at 24 or 72 hours after treatment. Values are means \pm SD for 5 rats. ** $p < 0.01$, in comparison to control.

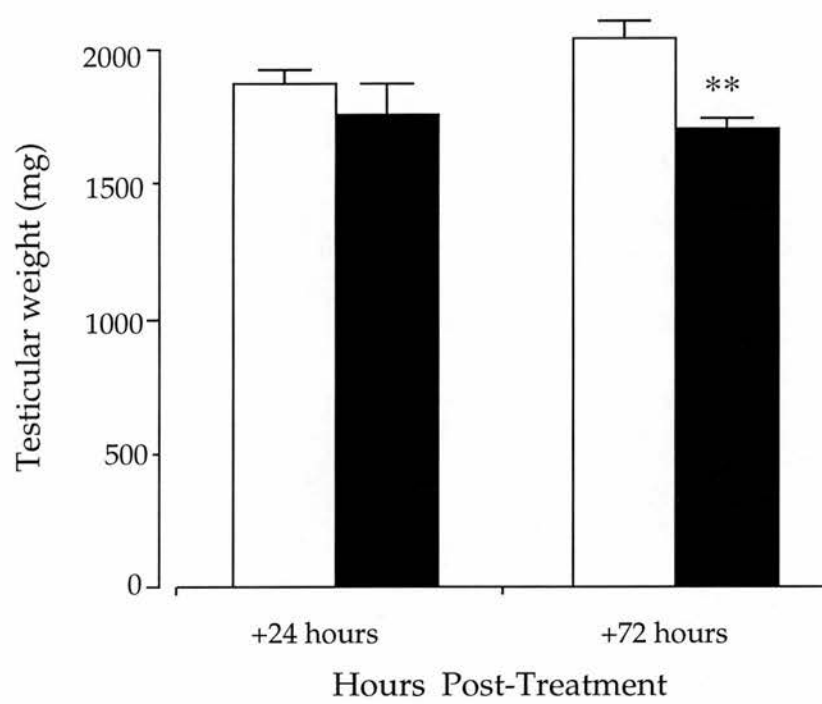


Figure 7.2. Stage-dependent changes in the incorporation of ^{35}S -methionine over 24 hours into newly synthesized proteins secreted *in-vitro* by seminiferous tubules isolated at different stages of the spermatogenic cycle from control rats (open bars) or rats pretreated with 650mg/kg MAA (toned bars) either 24 (top) or 72 (bottom) hours earlier. Values are means \pm SD for 3 rats per stage group. ** $p < 0.01$, *** $p < 0.001$, in comparison with respective control group.

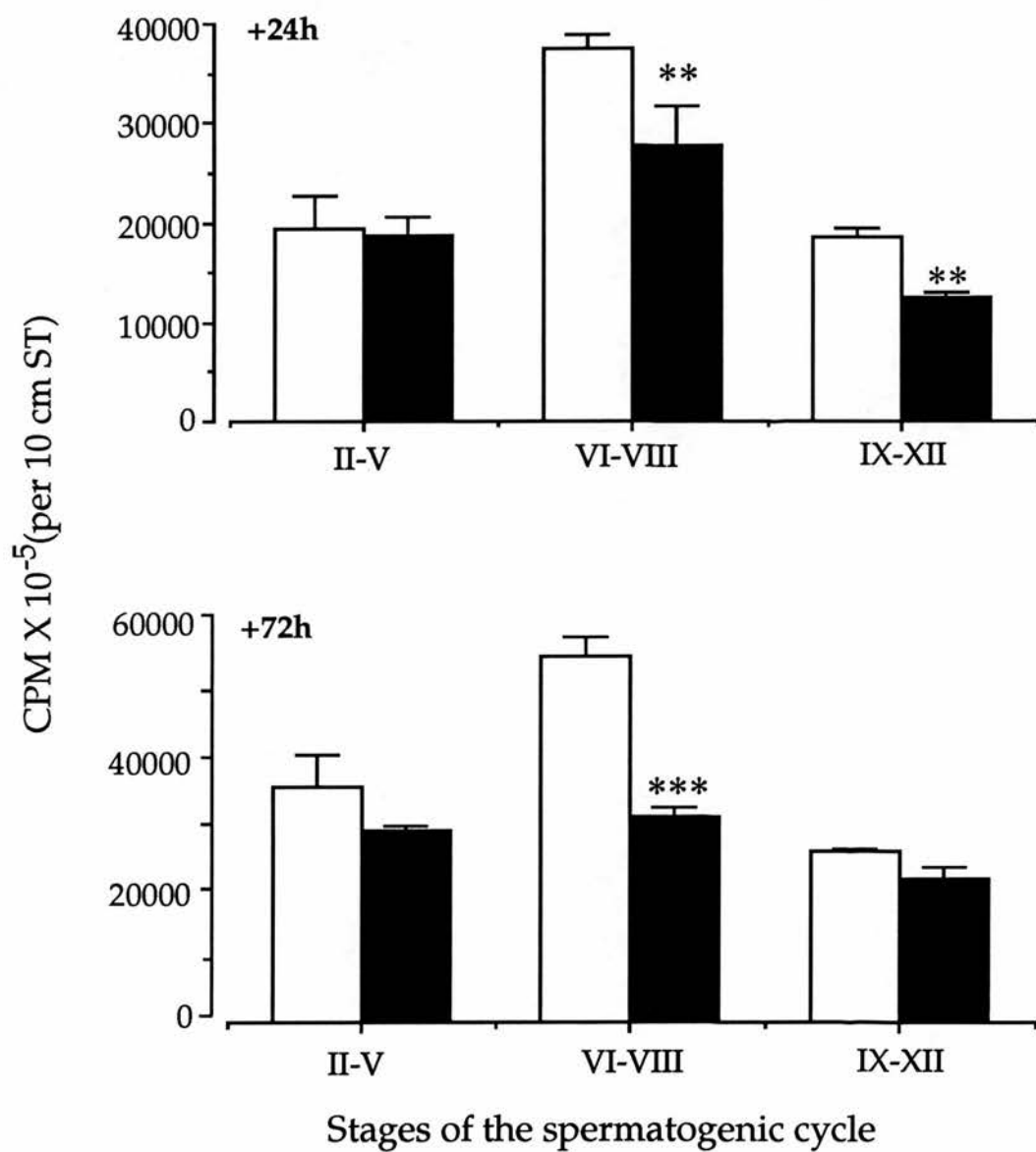
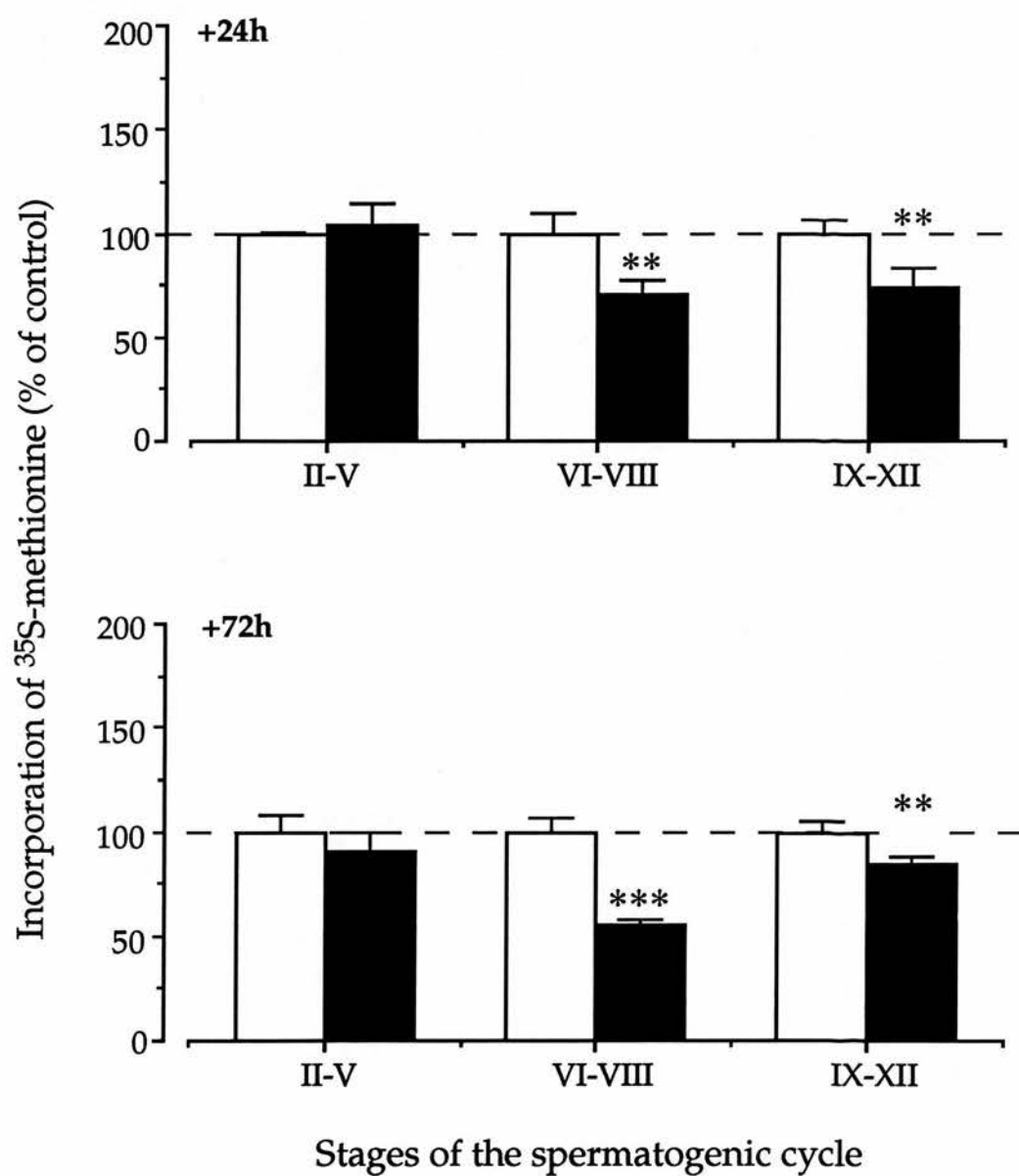


Figure 7.3. Incorporation of ^{35}S -methionine over 24 hours into newly synthesized proteins secreted *in-vitro* by seminiferous tubules isolated at different stages of the spermatogenic cycle from control rats (open bars) or rats pretreated with 650mg/kg MAA (toned bars) either 24 (top) or 72 (bottom) hours earlier. At each stage, incorporation has been expressed as a percentage of the mean control value (dashed line) obtained during the same experiment. ** $p < 0.01$, *** $p < 0.001$, in comparison with respective control group.



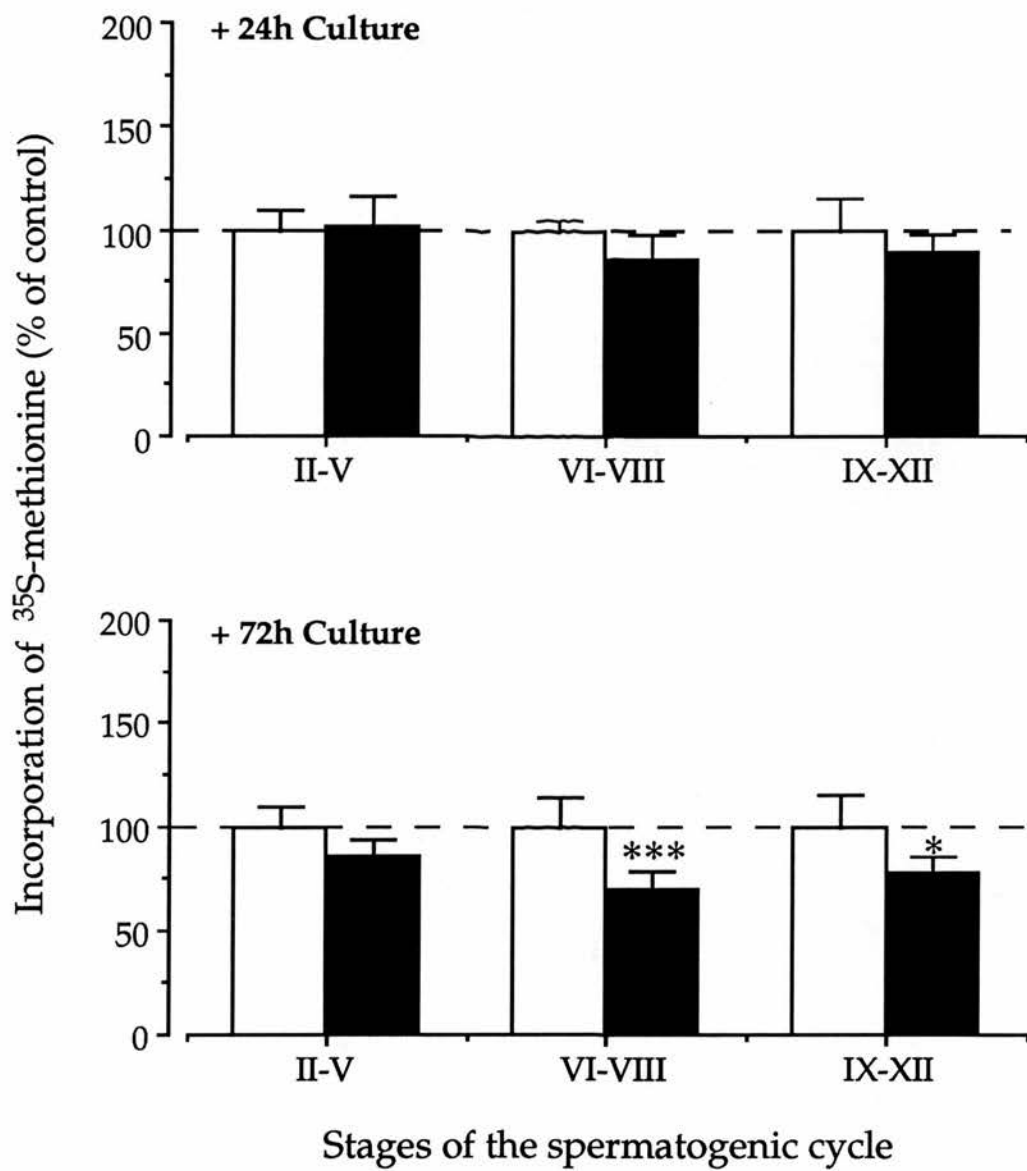
newly synthesized proteins secreted into the incubation medium by ST at stages VI-VIII and IX-XII when isolated 24 hours after treatment (Figure 7.3). ST at stages II-V showed no significant change in incorporation when compared with controls (Figure 7.3). By 72 hours after MAA treatment the decrease in incorporation of radiolabel into secreted proteins at stages VI-VIII was more pronounced ($p < 0.001$) and was comparable to the level of protein secretion found at stages II-V and IX-XII in controls (Figure 7.3). A similar picture was obtained with ST that had been isolated from control rats and cultured in the presence of 10^{-4} M MAA for 24 or 72 hours (Figure 7.4). ST at stages VI-VIII and IX-XII showed a slight, though not significant, decrease in the incorporation of ^{35}S -methionine into secreted proteins, whereas stages II-V showed no change in incorporation. After 72 hours in culture there was a significant decrease in the incorporation of radiolabel by ST at stages VI-VIII and IX-XIII. ST at stages II-V showed no significant change in incorporation when compared with controls.

7.3.4. Two-dimensional SDS PAGE

As the depletion of pachytene spermatocytes had the most marked effect on overall protein secretion at stages VI-VIII, newly synthesized proteins secreted at these same stages, from control and MAA-treated rats, were analysed by 2-D SDS PAGE (Figure 7.5). It can be seen that the majority of radiolabelled proteins were unaffected by exposure to MAA. Seven secreted proteins (numbered 1, 2, 3, 4, 6, 7, 8) were singled out on the basis that they showed changes in every experiment following exposure to MAA. A summary of the molecular weights and isoelectric points of these proteins is given in Table 7 and the position of each is circled and labelled on each of the gels.

The secretion of two of the identified proteins (numbered 2, 6 and 9) had a similar M_r to proteins which have been shown to be androgen regulated (Sharpe *et al.*, 1992) and germ cell dependent (McKinnell and Sharpe, 1992). The secretion of proteins numbers 1, 7 and 8 was found to be reduced slightly following administration of MAA 24 hours earlier. These three proteins are known to be secreted by ST at stages II-V and IX-XII in addition to being secreted by ST at stages VI-VIII and, following exposure to either m-DNB (see Chapter 5) or NB (see Chapter 6), they show pronounced reductions in abundance or are completely absent. Exposure to MAA 24 hours earlier resulted in a slight increase in the secretion of protein 3. The

Figure 7.4. Incorporation of ^{35}S -methionine over 24 hours into newly synthesized proteins secreted *in-vitro* by seminiferous tubules isolated at different stages of the spermatogenic cycle from untreated control rats and then cultured in the absence (open bars) or presence (toned bars) of 10^{-4}M MAA for either 24 (top) or 72 (bottom) hours. At each stage, incorporation has been expressed as a percentage of the mean control value obtained during the same experiment. Values are means \pm SD of $n=3$. * $p<0.05$, ** $p<0.01$, in comparison with respective control group.



Protein No	M _r (kDa)	pI	Effect of MAA on relative abundance	Stage-dependence of secretion	Possible Identity
1	63 - 68	5.6 - 5.8	Decrease	All stages	unknown
2	38 - 38.5	5.8 - 6.0	Decrease	VI-VIII	ARP-4 (Sharpe <i>et al.</i> , 1992)
3	28 - 30	5.8 - 6.9	Increase	All stages	Possible charge isomers of SGP-2
4	23 - 24	5.4 - 5.8	Decrease	All stages	unknown
6	25 - 30	6.9 - 7.4	Decrease	VI-VIII	ARP-3 (Sharpe <i>et al.</i> , 1992)
7	18 - 20	5.3 - 5.9	Decrease	All stages	unknown
8	17.5 - 20	5.9 - 6.4	Decrease	All stages	unknown

Table 7. List of seminiferous tubule-secreted proteins which showed repeatable differences in relative abundance following treatment *in-vivo* with MAA (650mg/kg). Protein numbers correspond to those shown in Figure 7.5.

Figure 7.5. Incorporation of ^{35}S -methionine into newly synthesized proteins secreted *in-vitro* by seminiferous tubules isolated at stages VI-VIII from control rats (top) or from rats treated 24 hours earlier with MAA (650mg/kg) (bottom). Proteins which change in relative abundance following MAA exposure are circled and numbered. The positions of the three major Sertoli cell secreted proteins, sulphated glycoproteins-1 (SGP-1), sulphated glycoprotein-2 (SGP-2) and cyclic protein-2 (CP-2), are indicated for reference.

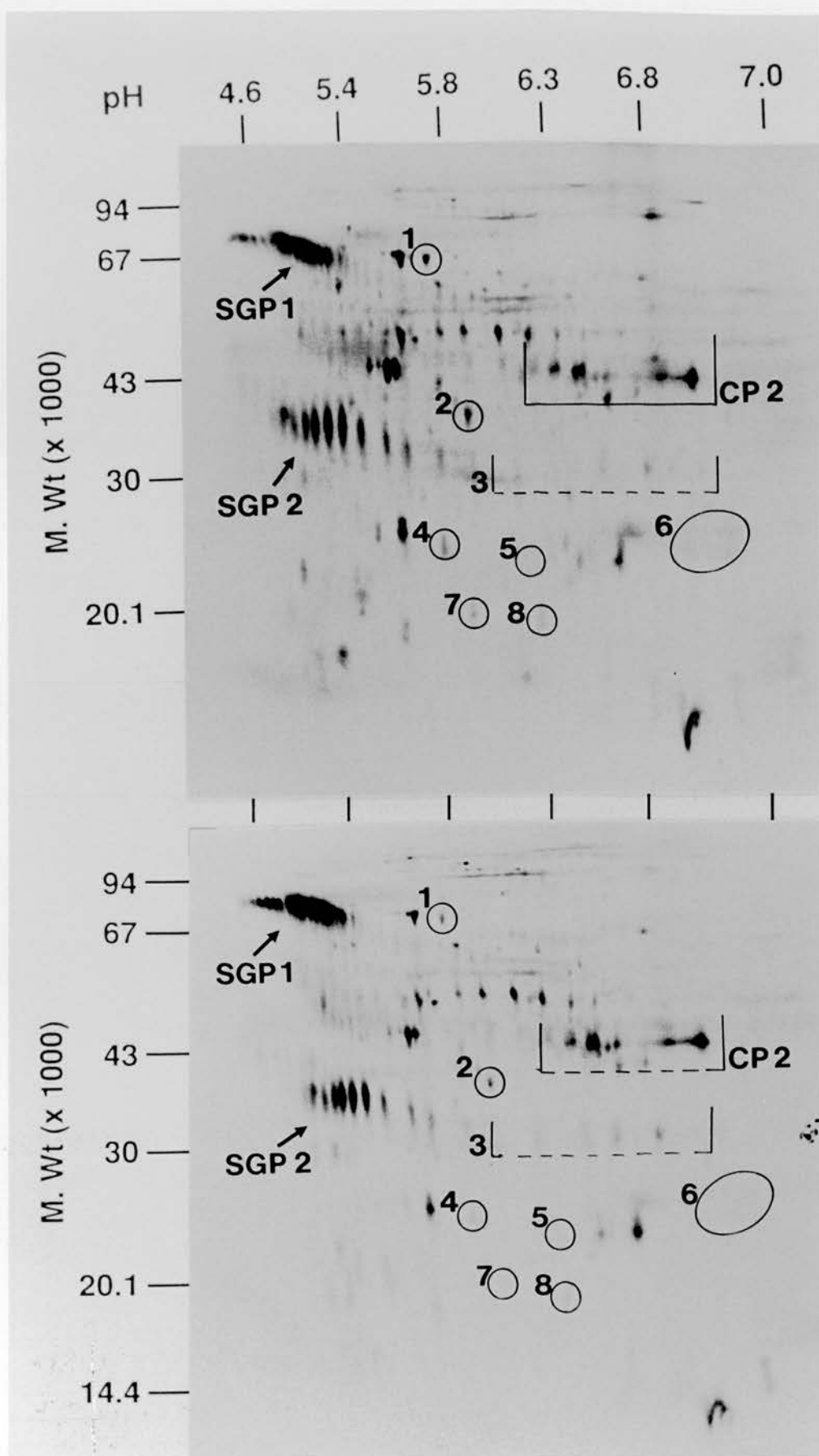
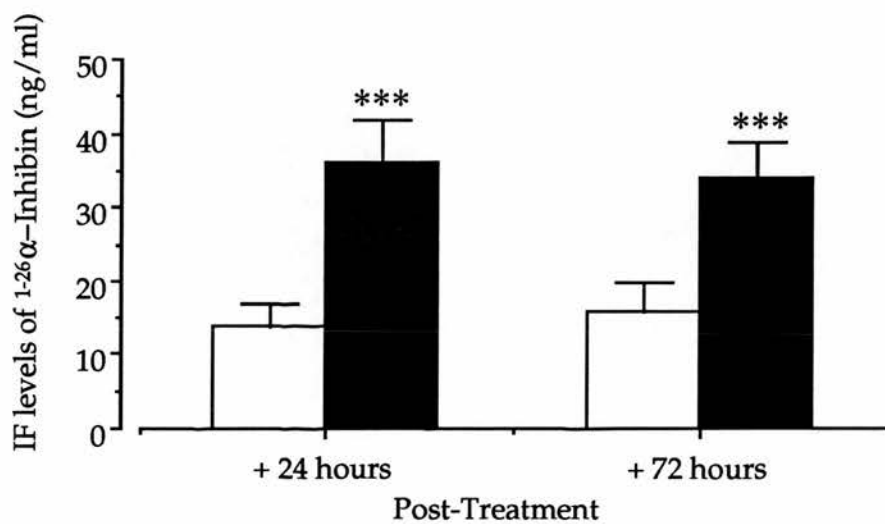
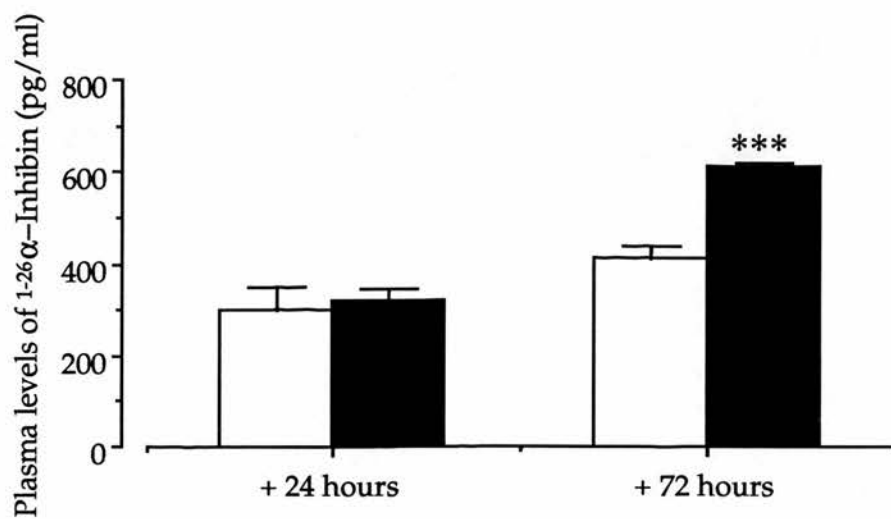


Figure 7.6. Effect of a single oral administration of MAA (650mg/kg) (toned bars) or vehicle alone (2.9mls/kg) (open bars) on levels of immunoactive inhibin in plasma (top) or testicular interstitial fluid (IF) (bottom) collected from rats at 24 or 72 hours after administration. Values are means \pm SD for 5 rats. *** $p < 0.001$ in comparison with respective control group.



identity of this protein is unknown, although it is possible that it represents isomers of SGP-2 with a high pI.

7.3.5. Effect of MAA on levels of inhibin in plasma and IF

The levels of immunoactive inhibin in plasma and testicular interstitial fluid (IF) were increased significantly at 1 and 3 days after treatment with MAA ($p < 0.001$), when compared to levels in control rats (Figure 7.6).

7.4. Discussion

The objective of the experiments described in this chapter was to assess the early effects of selective germ cell depletion, induced by MAA- treatment, on seminiferous tubule protein secretion at the different stages of the spermatogenic cycle. In addition, testicular weight and the secretion of immunoactive inhibin were used as indices of testicular dysfunction.

In the present study, a single oral administration of MAA at a dose known to selectively destroy 80-100% of pachytene and later spermatocytes (Bartlett *et al.*, 1988; Sharpe, 1989; Allenby *et al.*, 1991a; Sharpe *et al.*, 1991) at all stages other than early-to mid-stage VII, of the spermatogenic cycle, resulted in stage-specific changes in the overall incorporation of ^{35}S -methionine into proteins secreted by ST isolated at 24 and 72 hours after treatment with MAA. When compared with controls, the overall level of incorporation of radiolabel into proteins secreted by seminiferous tubules at stages VI-VIII or IX-XII was reduced significantly ($p < 0.01$) when isolated 24 hours after treatment. There was no significant change in incorporation at stages II-V. By 72 hours after treatment with MAA, the decrease in incorporation of radiolabel into secreted proteins at stages VI-VIII was more pronounced ($p < 0.001$) and was comparable to the level of protein secretion found at stages II-V and IX-XII in controls (Figure 7.3); a similar profile of incorporation was found in staged tubules that had been isolated from untreated control animals and cultured for 24 or 72 hours in the presence of MAA (10^{-4}M), a dose equivalent to the calculated peak blood levels obtained *in-vivo* following a single oral dose of 500mg/kg bodyweight (P.M.D. Foster – personal communication).

In this and previous studies (Sharpe *et al.*, 1992) the overall level of incorporation of ^{35}S -methionine into proteins secreted by seminiferous

tubules at stages VI-VIII of the spermatogenic cycle from control rats was approximately double that seen at stages II-V or IX-XII (Figure 7.2). Based on studies which have used ethane dimethane sulphonate (EDS) to induce testosterone withdrawal, Sharpe *et al.* (1992) established that the increase in protein secretion which occurs normally at stages VI-VIII is completely androgen-dependent. Depletion of pachytene spermatocytes in seminiferous tubules by MAA administration 24 hours earlier prevented partially the increase in protein secretion which occurs normally at stages VI-VIII (Figure 7.2). By 72 hours after MAA treatment the increase in protein secretion at stages VI-VIII was prevented completely (Figure 7.2). Moreover, this reduction occurred without any corresponding significant change in the circulating or intratesticular levels of testosterone (Bartlett *et al.*, 1988; Allenby, 1990). From the results of these studies it may be suggested that pachytene spermatocytes must be present if the normal (androgen-dependent) increase in protein secretion is to occur when tubules progress from stages II-V to VI-VIII.

The susceptibility of pachytene spermatocytes to the effects of MAA varies markedly according to the stage of the spermatogenic cycle, with pachytene spermatocytes at early- to mid-stage VII being highly resistant to the adverse effects of MAA (Bartlett *et al.*, 1988) and it may be assumed that this subset of pachytene spermatocytes is responsible for the slight increase in protein secretion at stages VI-VIII which still occurred following MAA treatment 24 hours earlier (Figure 7.2). Based on the incorporation of ³⁵S-methionine into overall secreted proteins it would seem that the depletion of pachytene spermatocytes has little effect on seminiferous tubule protein secretion at stages II-V or IX-XII.

MAA is thought to act on pachytene spermatocytes, rather than on Sertoli cells, and it is thought that MAA exerts its effects by altering energy metabolism through inhibition of the enzyme lactate dehydrogenase - C4 (Williams and Foster, 1987). Since pachytene spermatocytes have the highest specific activity of this enzyme (Meistrich *et al.*, 1977) it is possible that such an inhibition may be a contributory feature of the testicular toxicity of MAA. In this respect MAA treatment has been shown previously to cause mitochondrial swelling in pachytene spermatocytes (Foster *et al.*, 1983). More recently, Spano *et al.* (1991) reported a change in the mitochondrial mass distribution of the round spermatids that survive MAA treatment. It is not known why a subset of pachytene spermatocytes should remain resistant to MAA, but it is perhaps significant that these germ cells have extremely high

levels of expression of the gene that encodes for cytochrome oxidase II (Saunders *et al.*, 1993). Cytochrome oxidase is the terminal enzyme in the electron transport chain located on the inner mitochondrial membrane, and its function is to generate adenosine triphosphate (ATP), which provides the energy required by cells for their cellular processes. Thus, the increased capacity of pachytene spermatocytes at stage VII to generate energy may in some way protect these cells against MAA-induced changes in energy metabolism.

Analysis of ST-secreted proteins by 2-D SDS PAGE identified seven proteins which showed reproducible changes in protein secretion following exposure to MAA; in most cases (proteins numbered 1, 2, 4, 6, 7 and 8 in Figure 7.5) their secretion was reduced. The finding that the secretion of proteins numbered 1, 2, 4, 6, 7 and 8 was reduced only slightly by MAA treatment (in comparison to the effects of m-DNB and NB; Chapters 5 and 6) may be due to the small population of pachytene spermatocytes at early- to mid-stage VIII which are resistant to the effects of MAA and as such might be able to maintain the secretion of these proteins at marginally subnormal levels. Two of these proteins (numbered 2 and 6) correspond in molecular weight and pI to two recently identified stage-specific androgen regulated proteins (Sharpe *et al.*, 1992) which a subsequent study has shown are germ cell dependent (McKinnell and Sharpe, 1992). Exposure to MAA 24 hours earlier resulted in a slight increase in the secretion of protein 3. The identity of this protein is unknown, although it is possible that it represents isomers of SGP-2 with a high pI. The identity of proteins 1, 4, 7 and 8 is unknown.

A comparison of the 2-D profile of ST-secreted proteins following exposure to either m-DNB, NB or MAA revealed that the secretion of the seven proteins, which this study has identified, was affected similarly following exposure to either m-DNB (see Chapter 5) or NB (see Chapter 6). m-DNB and NB alter Sertoli cell function initially, and cause the loss/degeneration of pachytene spermatocytes in ST at stages VII-XII, as a secondary event, whereas MAA causes the selective depletion of pachytene spermatocytes at all stages other than early- to mid-stage VII. The fact that proteins numbered 1, 2, 6, 7 and 8 were affected in a similar way following all three treatments, while pachytene spermatocytes were either absent from the ST or degenerating, suggests either that these proteins might derive from pachytene spermatocytes or that they are secretory products of Sertoli cells, the secretion of which is dependent on the presence of pachytene spermatocytes. The secretion of protein 3, shown in this study to increase

slightly following exposure to MAA, has been shown to be increased substantially following exposure to either m-DNB or NB (see Chapter 5 and 6, respectively). The secretion of this protein has been shown to be prominent in Sertoli cell+germ cell co-cultures from immature rats, still prominent but less so in ST from immature rats and absent or of very low abundance in the adult (see Chapter 4). Taken together, these results suggest that the secretion of protein 3 is increased in the absence of germ cells, and this suggests that germ cells are capable of modulating the secretory function of the Sertoli cells and, perhaps, other germ cell types in the seminiferous epithelium (Jégou *et al.*, 1992; Sharpe *et al.*, 1992). Furthermore, the finding that removal of two generations of germ cells from the ST can also lead to a substantial increase in the secretion of this protein (Sharpe and McKinnell, unpublished data) indicates that its secretion is regulated by more than one germ cell type.

Following exposure to either m-DNB or NB the secretion of a 26 kDa protein (protein 5) could be induced (see Chapters 5 and 6, respectively). Interestingly, the secretion of this protein was not induced following exposure to MAA. These results therefore suggest that protein 5 might be a specific marker of Sertoli cell dysfunction. Exposure to MAA resulted in a slight decrease in the secretion of protein number 4, whereas exposure to either m-DNB or NB caused a marked decrease in the secretion of this protein (see Chapters 5 and 6, respectively). Protein 4 has been shown by McKinnell and Sharpe (1992) not to be secreted when round spermatids are selectively depleted from the seminiferous tubule. However, at 24 hours after MAA treatment, round spermatids are known to be present in the seminiferous tubule (Foster *et al.*, 1983, 1984, 1987; Creasy *et al.*, 1985; Bartlett *et al.*, 1988), although a recent study has shown a change in the mitochondrial mass distribution of those which survive MAA treatment (Spano *et al.*, 1991). It has yet to be established therefore whether protein 4 originates from the Sertoli cell and/or round spermatids as the results presented here do not distinguish whether reduction of this protein is caused by (a) a primary change in the secretory function of the Sertoli cell, resulting in the loss of round spermatids which are the source of the protein or (b) secondary changes in Sertoli cell and/or germ cell secretory function as a consequence of depletion of pachytene spermatocytes, following exposure to MAA.

In this study, coincident with pachytene spermatocyte degeneration, major changes were observed in the secretion of immunoactive inhibin in plasma and testicular IF following treatment with MAA 3 days and, to a lesser extent, 1 day earlier (Figure 7.6). Similarly, blood levels of FSH have

been shown previously to follow a similar pattern of change (Allenby *et al.*, 1991a). It is not possible to make a definite conclusion as to the cause of the increase in the secretion of immunoactive inhibin 1-3 days after MAA treatment, but this could be due to a direct stimulatory effect of MAA on the Sertoli cell or to the depletion of pachytene spermatocytes having an indirect effect on Sertoli cell function. These results confirm those of a previous study by Allenby *et al.* (1991a) and suggest that germ cells are capable of modulating Sertoli cell function. However, the use of inhibin as a marker of disruption to spermatogenesis is limited as it is known that inhibin and inhibin-related peptides are produced by extratesticular sources (Meunier *et al.*, 1978), which probably limits its usefulness.

In conclusion, the experiments presented in this chapter have shown that MAA causes stage-specific changes in the secretion of proteins by isolated ST and has identified seven proteins which have potential use as markers of MAA-induced damage to spermatogenesis. In addition, it has been shown that the androgen-dependent increase in overall protein secretion by seminiferous tubules at stages VI-VIII of the spermatogenic cycle is dependent upon the presence of pachytene spermatocytes and that the modulatory effect of germ cells on seminiferous tubule protein secretion varies according to the stage of the spermatogenic cycle and thus of the germ cell complement.

8. Effect of local heating on ST protein secretion

Chapters 5 and 6 described the adverse testicular effects of two Sertoli cell toxicants on seminiferous tubule protein secretion and identified stage-specific changes in overall protein secretion. Analysis by 2-D SDS PAGE identified ten potential marker proteins of toxicant-induced disruption to spermatogenesis. Of particular interest was the finding that, following exposure to either meta-dinitrobenzene or nitrobenzene, the secretion of a 26kDa protein (protein 5) could be induced. In Chapter 7, the effect of selective pachytene spermatocyte depletion on seminiferous tubule protein secretion was assessed. Stage-specific changes in overall protein secretion, similar to those observed following exposure to meta-dinitrobenzene and nitrobenzene, were observed following exposure to methoxyacetic acid. Analysis by 2-D SDS PAGE identified seven proteins that were associated with the loss of pachytene spermatocytes; interestingly, the secretion of protein 5 was not induced by exposure to methoxyacetic acid. These results therefore suggest that the secretion of protein 5 might be a specific marker of Sertoli cell dysfunction. In this chapter, the effects of severe disruption of spermatogenesis, induced by short-term local testicular heating, were assessed in order to establish whether protein changes comparable to those seen following toxicant exposure could be induced.

8.1. Introduction

The adverse effects of heat on the mammalian seminiferous epithelium are now well documented. Early work on natural and experimental cryptorchidism in guinea pigs and rats (Moore, 1924a) and in sheep (Moore and Oslund, 1923) led to the conclusion that normal spermatogenesis in male mammals with a scrotum requires a temperature lower than body temperature. At this time also, Fukui (1923) and Moore (1924b) showed independently that local heating of the testis could induce histological changes similar to those induced by experimental cryptorchidism. In fact, it has been shown that temperatures above 37°C are effective in inducing histological change in most mammals, and that the higher the temperature, the shorter is the exposure needed to induce a similar degree of histological change (Moore and Chase, 1923; Young, 1927; Asdell and Salisbury 1941; Nelson, 1951). The histological changes described in these early studies were mainly descriptive, which led to contradictory conclusions, and failed to

resolve the problem of the heat susceptibility of specific types of cells of the seminiferous epithelium.

More recently, quantitative studies of changes in the seminiferous epithelium of rats exposed to either experimental cryptorchidism (Clegg, 1963) or short-term local testicular heating (Steinberger and Dixon, 1959) have been undertaken. For example, Clegg (1963) found that cryptorchidism led to degenerative changes in the seminiferous tubules which were maximal after 15 days, after which a limited regeneration occurred. Clegg found also that spermatogonia were the least affected cells, although meiosis and mitosis were inhibited partially, whilst Steinberger and Dixon (1959) concluded that, following exposure to temperatures of 43°C for 15 minutes, primary spermatocytes are the germ cells most sensitive to damage by heat. Subsequently, Chowdhury and Steinberger (1964) established that not all spermatocytes were equally susceptible to the adverse effects of heat and, on the basis of their results, susceptible cells can be placed into 3 categories; 1) cells which disappear from the seminiferous epithelium within 2 days of exposure (spermatids at step I of spermiogenesis; pachytene spermatocytes in stages VII-XII and the diakinetik and dividing spermatocytes in stages XIII-XIV); 2) cells which are apparently capable of some differentiation into more advanced stages before degeneration and disappearance (zygotene spermatocytes in stage XIII and pachytene spermatocytes in stages I-IV); and 3) cells which are capable of considerable development and differentiation, including division and formation of spermatids. These spermatids, however, disappear within 8 to 26 days. A later study by Chowdhury and Steinberger (1970) extended their previous findings by showing that morphological changes could be observed within 1 hour of exposure to short-term local testicular heating (43°C for 15 minutes). These changes were detected in pachytene spermatocytes in stages IX-XII, the diakinetik and dividing spermatocytes in stages XIII-XIV and the young spermatids at step I of spermiogenesis. Within 4 hours of exposure to heat, the damage could also be detected in pachytene spermatocytes at stages VII-XIV. The pachytene, diakinetik and dividing spermatocytes from stages IX-XIV were absent 24 hours after exposure, whilst those at stages VII and VIII showed a drastic reduction in number. Complete recovery from moderate heating is, however, usually evident in the rat by 56 days after exposure and thereafter, as judged by testis weight (Jégou *et al.*, 1984).

In addition to germ cell degeneration, increased temperatures have been shown also to have adverse effects on the morphology and functions of

the Sertoli cell, as evidenced by an increased rate of phagocytosis, accumulation of lipid droplets (Clegg, 1963), a modification of inter-Sertoli cell junctional complexes (Kerr *et al.*, 1979) and a reduction in cytoplasmic organelles (Aumüller *et al.*, 1980). Available evidence suggests also that, following severe germ cell depletion induced by cryptorchidism, overall secretion by the Sertoli cell of seminiferous tubule fluid, inhibin and ABP is reduced drastically at certain times after treatment (Hagenas and Ritzén, 1976; Au *et al.*, 1983; Jégou *et al.*, 1983). Moderate but selective heating of the testis appears to have comparable effects (Jégou *et al.*, 1984; Au *et al.*, 1987). Such data have been interpreted as evidence for a general decrease in Sertoli cell secretory function following disruption of spermatogenesis. The results of a study by Sharpe and Bartlett (1987) suggest, however, that the situation may not be quite so simple. The finding of increased levels of ABP in interstitial fluid, following short-term local heating (43°C for 30 minutes) or by unilateral cryptorchidism, suggests that there has been a major change in the direction of secretion of ABP, as it is clear from studies using short-term unilateral efferent duct ligation that ABP secretion into the tubule lumen is decreased considerably (Hagenas and Ritzén, 1976; Jégou *et al.*, 1984). These results suggest that the absolute rate of production of ABP by the Sertoli cell is decreased following disruption of spermatogenesis, but that a greater proportion than normal is secreted via the base of the Sertoli cell into testicular interstitial fluid and thence into the blood stream. The physiological significance of this change remains to be established.

As Sertoli cells are known to regulate testicular interstitial fluid volume, and as germ cells are thought to modulate Sertoli cell function, Sharpe *et al.* (1990) assessed whether depletion of specific germ cell types *in-vivo* is associated with changes in recovered IF volume. Germ cell depletion was induced either by short-term local testicular heating (43°C for 30 minutes) or by a single administration of the germ cell specific toxicant, methoxyacetic acid (650mg/kg). The results of this study provided evidence that specific depletion of the most mature germ cell type (the elongate spermatids) is associated with specific changes in testicular IF volume, presumably via modulation of the secretion of vasoactive factors by the Sertoli cells.

Available evidence indicates also the existence of Sertoli cell-Leydig cell interactions. Using heat exposure (43°C for 15 minutes) to induce disruption of spermatogenesis, Jégou *et al.* (1984) found changes in Leydig cell function which were separated in time from the actual thermal stress, but

which coincided with the depletion of elongate spermatids. The authors concluded that the changes observed were the result of initial changes in Sertoli cell function, secondary to disruption of germ cell function. Further support for the existence of Sertoli cell-Leydig cell interactions was provided by Bartlett and Sharpe (1987). Using short-term exposure of the rat testis to heat, the effects of moderate (43°C for 15 minutes) and severe (43°C for 30 minutes) disruption to spermatogenesis on the amounts of an interstitial fluid factor or factors (which stimulate or stimulates Leydig cell testosterone production *in-vitro*) were assessed. It was found that, after exposure to heat for 15 minutes, no significant change in interstitial fluid activity, with respect to controls, was observed. In contrast, after exposure to heat for 30 minutes bioactivity was increased significantly within 3 days of treatment, and thereafter remained elevated throughout the period of observation. Levels of ABP in interstitial fluid showed a similar pattern of change. The authors concluded that the parallel changes in activity of the interstitial fluid factor(s) and concentrations of ABP in interstitial fluid provide evidence that these products are derived from the Sertoli cell.

The objective of the experiments described in this chapter was to assess whether reproducible stage-specific changes in the secretion of proteins by isolated ST could be identified following short-term local testicular heating.

8.2. Experimental Procedures

8.2.1. Local testicular heating

Young adult male rats aged approximately 70 days were anaesthetized with an i.p. injection of 50mg sodium pentobarbitone/kg (Sagatal; May and Baker Ltd, Dagenham, Essex, UK). The lower half of their body, including the scrotum, was then immersed for 30 minutes in a thermostatically controlled water bath maintained at 43°C, whilst the animals were strapped with velcro ties to a supporting frame. This treatment has been shown previously to cause widespread early germ cell loss, (i.e. pachytene spermatocytes and early spermatids) although still in a stage-specific and time-related manner (Bartlett and Sharpe, 1987).

8.2.2. Perfusion-fixation of control and heat-treated rats

At 24 hours after treatment 2 rats were perfusion-fixed according to the methods described in Chapter 3 and data compared to data for 2 perfusion fixed control rats.

8.2.3. Procedure for isolation of ST from control and heat-treated rats

At 4 and 24 hours after treatment, seminiferous tubules from control and heat-treated rats were isolated and cultured according to the methods described in Chapter 3.

8.2.4. Measurement of newly synthesized proteins

Incorporation of ^{35}S -methionine into both secreted and intracellular proteins was determined by precipitation of aliquots of culture medium or cell lysates with 10% (v/v) trichloroacetic acid, according to the methods described in Chapter 3.

8.2.5. Two-dimensional SDS PAGE

Newly synthesized ^{35}S -methionine labelled proteins were evaluated according to the methods described in Chapter 3. ST conditioned medium from control and heat-treated rats from the same experiment were always run in parallel on SDS PAGE, and subsequent determination of any change in the relative abundance of proteins on the gels was by comparison of gels from treated animals with their respective control from the same experiment. There was little variation between gels from different experiments.

8.3. Results

8.3.1. Effect of local testicular heating on testicular morphology

In rats exposed to 43°C for 30 minutes, examination of sections from perfusion-fixed testes (Figures 8.1 and 8.2) confirmed in general the detailed analyses of Chowdhury and Steinberger (1964, 1970). In such testes, selective and stage-specific damage to pachytene spermatocytes was the most obvious change (II-XIV), whilst the nuclei of step 16 spermatids were found to be embedded deeply in the Sertoli cell cytoplasm at stages VI-VIII as opposed to

Figure 8.1. Representative morphology of seminiferous tubules isolated at different stages of the spermatogenic cycle from control rats (top) showing pachytene spermatocytes (large arrowheads), round spermatids (small arrowheads) and the heads of elongate spermatids (small arrows) or from rats which had been exposed to short-term local testicular heating 24 hours earlier (bottom) showing degenerating pachytene spermatocytes (large arrowheads). Scale bars = 300 μ m.

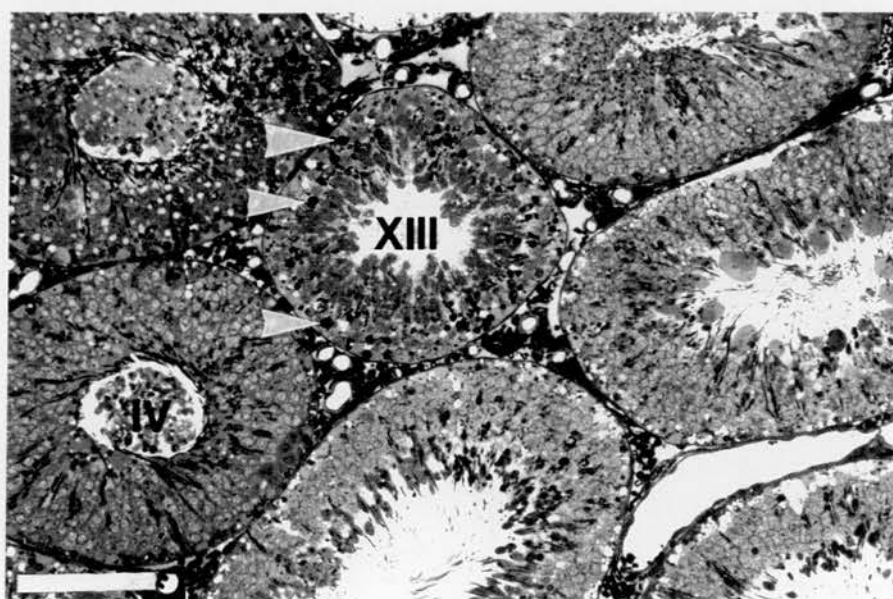
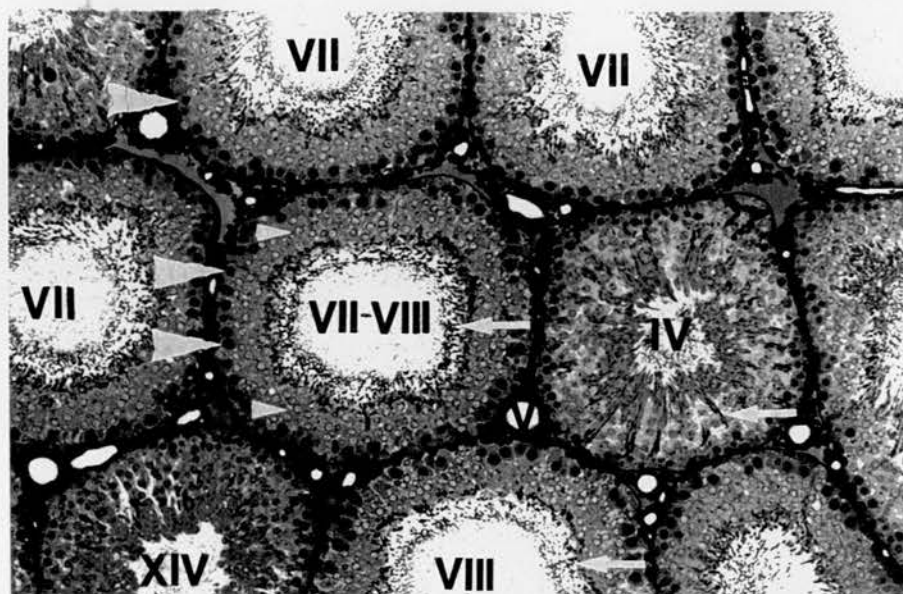
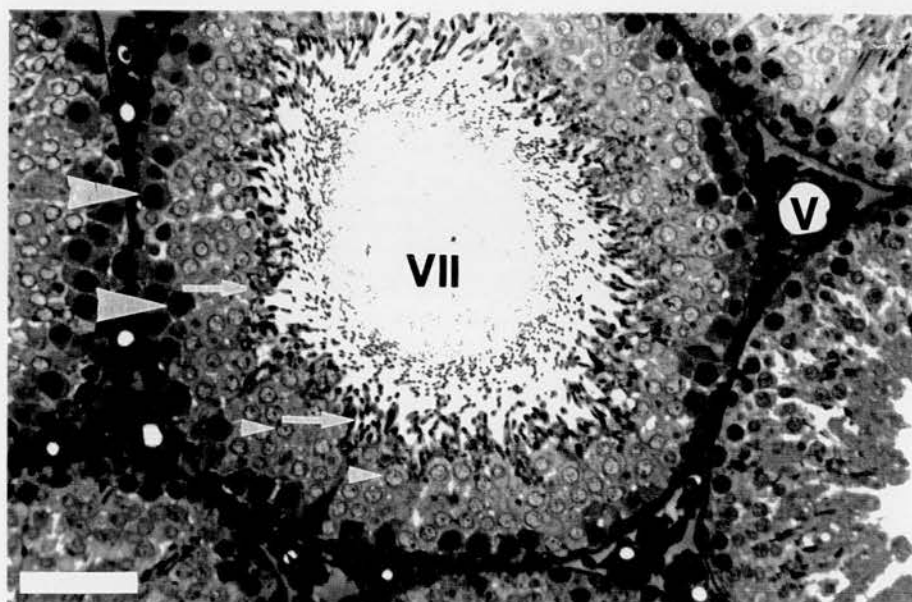


Figure 8.2. Representative morphology of a stage VII seminiferous tubule isolated from control rats (top) showing pachytene spermatocytes (large arrowheads), round spermatids (small arrowheads) and the heads of elongate spermatids (small arrows) or from rats which had been exposed to short-term local testicular heating 24 hours earlier (bottom) showing degenerating pachytene spermatocytes (large arrowheads), normal spermatids (small arrowheads) and the heads of elongate spermatids (small arrows) embedded deeply in the Sertoli cell cytoplasm. The interstitial space (S) and blood vessels (V) are shown also. Scale bars = 100 μ m.



being grouped around the edge of the tubule lumen (Figure 8.1). Large vacuoles in the epithelium of many tubules were also evident (Figure 8.2). Round spermatids in stages I-VII appeared normal in comparison to controls.

8.3.2. Effect of local testicular heating on testicular weight

Mean testicular weight was reduced significantly ($p < 0.001$) when compared with controls at 24 hours after local testicular heating but not at 4 hours after treatment (Figure 8.3).

8.3.3. Stage-dependent differences in overall protein secretion

The overall level of incorporation of ^{35}S -methionine into proteins secreted into the incubation medium by ST at stages VI-VIII from control rats was more than double that at stages II-V or IX-XIII whereas incorporation into intracellular proteins showed no such difference between the stages (Figure 8.4). This difference was evident in the control group in each of the experiments described below. In view of this and differences between experiments in the actual level of incorporation of ^{35}S -methionine into ST-secreted and intracellular proteins, data has been normalised by expressing incorporation as a % of the mean control value for that stage.

8.3.4. Effect of local testicular heating on overall protein secretion

Following short-term local testicular heating there was a significant increase ($p < 0.001$) in the incorporation of ^{35}S -methionine into newly synthesized proteins secreted into the incubation medium by ST at stages VI-VIII when isolated 4 hours after treatment (Figure 8.5); ST at stages II-V or IX-XII showed no significant change in incorporation when compared with controls (Figure 8.5). In contrast ST isolated from rats 24 hours after treatment showed a significant decrease in incorporation of radiolabel into secreted proteins at stages VI-VIII and IX-XII ($p < 0.001$ and $p < 0.05$, respectively, Figure 8.5); ST at stages II-V showed no change in incorporation at this time.

8.3.5. Effect of local testicular heating on synthesis of intracellular proteins

Following short-term local testicular heating there was a significant increase ($p < 0.01$) in the incorporation of ^{35}S -methionine into intracellular proteins in ST at stages VI-VIII when isolated 4 hours after treatment (Figure 8.5),

Figure 8.3. Effect of short-term local testicular heating (43°C for 30 minutes) (toned bars) on testicular weight 4 or 24 hours later. Values are means \pm SD for 5 rats. ** $p < 0.01$, in comparison with respective control group.

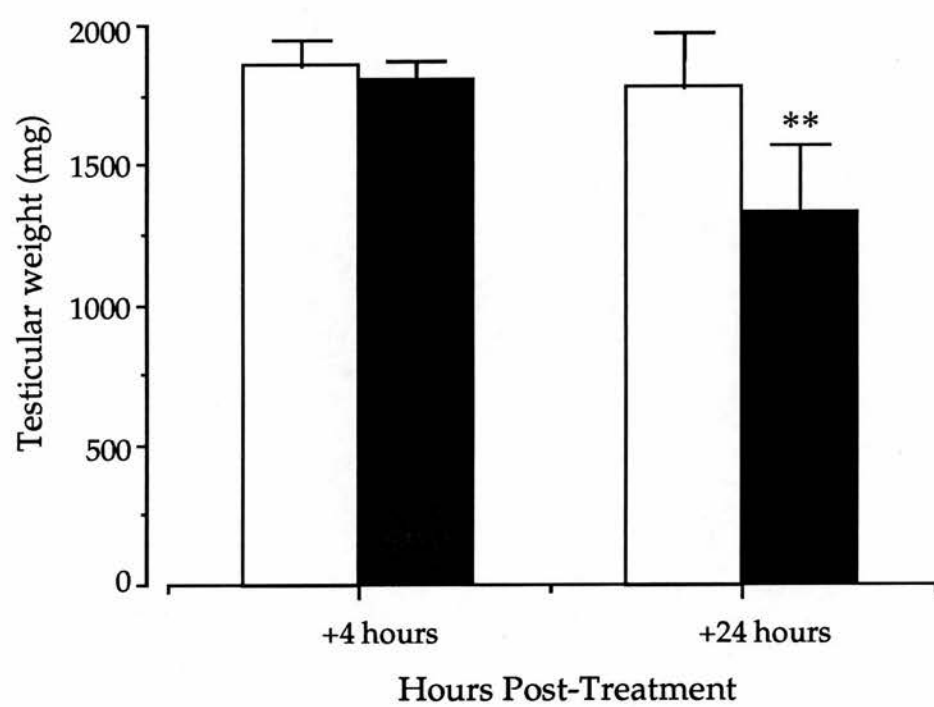


Figure 8.4. Incorporation of ^{35}S -methionine over 24 hours into newly synthesized secreted (top) or intracellular (bottom) proteins by seminiferous tubules isolated at different stages of the spermatogenic cycle from control rats (open bars) or rats exposed 24 hours earlier to short-term local testicular heating (43°C for 30 minutes) (toned bars). Values are means \pm SD for three rats per stage group. * $p < 0.05$, *** $p < 0.001$, in comparison with respective control group.

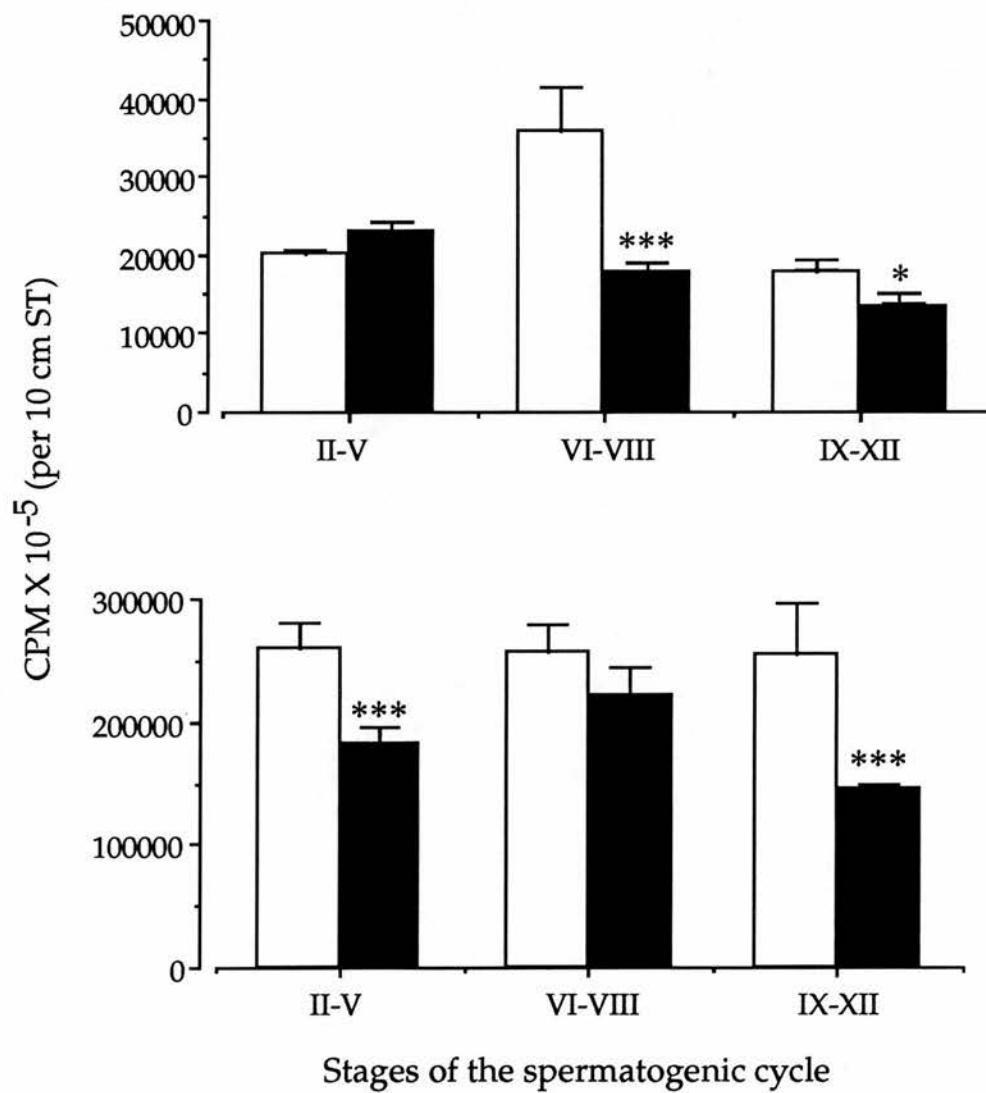


Figure 8.5. Incorporation of ^{35}S -methionine over 24 hours into newly synthesized proteins secreted *in-vitro* by seminiferous tubules isolated at different stages of the spermatogenic cycle from control rats (open bars) or rats exposed to short-term local testicular heating (43°C for 30 minutes) (toned bars) either 4 (top) or 24 (bottom) hours earlier. At each stage incorporation has been expressed as a percentage of the mean control value (dashed line) obtained during the same experiment. Values are means \pm SD of $n=4$. ** $p<0.01$, *** $p<0.001$, in comparison with respective control group.

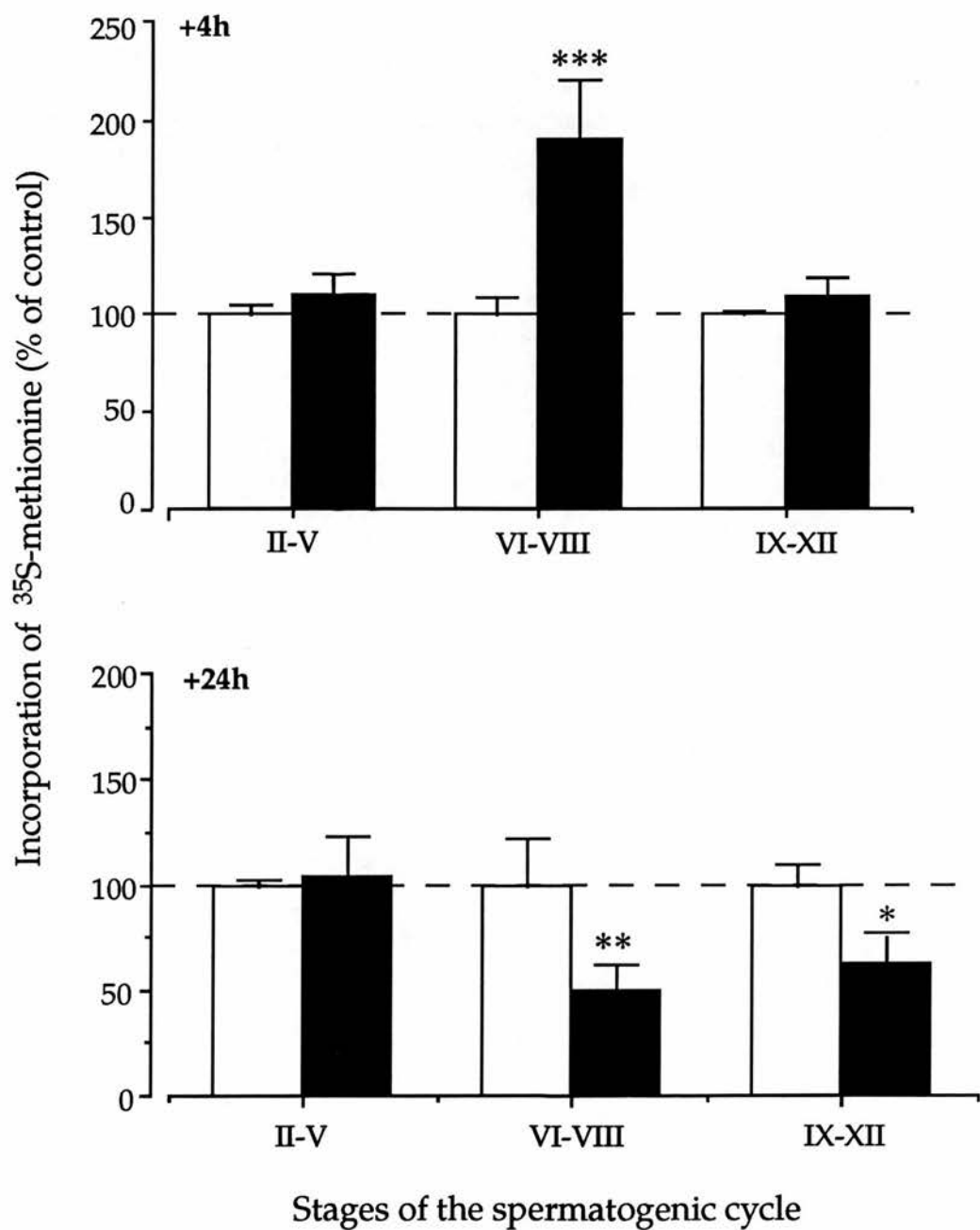
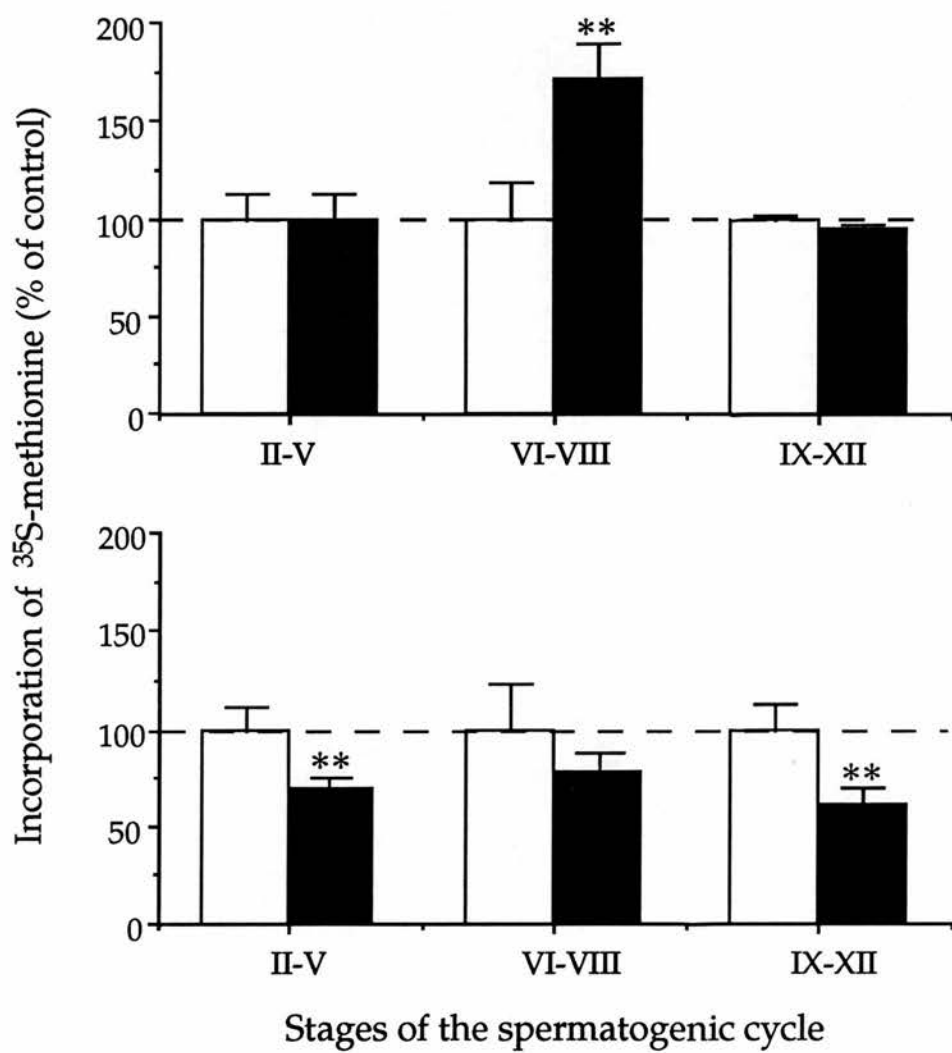


Figure 8.6. Incorporation of ^{35}S -methionine over 24 hours into newly synthesized intracellular proteins in seminiferous tubules isolated at different stages of the spermatogenic cycle from control rats (open bars) or rats exposed to short-term local testicular heating (43°C for 30 minutes) (toned bars) either 4 (top) or 24 (bottom) hours earlier. At each stage, incorporation has been expressed as a percentage of the mean control value (dashed line) obtained during the same experiment. Values are means \pm SD of $n=4$. ** $p<0.01$, in comparison with respective control group.



whereas ST at stages II-V or IX-XII showed no significant change in incorporation when compared with controls (Figure 8.6). By 24 hours after treatment the incorporation of radiolabel into intracellular proteins was decreased in ST at stages II-V, VI-VIII and IX-XII, although the decrease at stages VI-VIII was not significant statistically.

8.3.6. Two-dimensional SDS PAGE

As the effect of local testicular heating had the most marked effect on overall protein secretion at stages VI-VIII, newly synthesized proteins, secreted at these same stages, from control and heat-treated rats, were analysed by 2-D SDS PAGE (Figures 8.7 and 8.8). Following heat treatment 4 hours earlier, no major changes in the secretion of specific proteins were observed (Figure 8.7). However, by 24 hours after treatment seven secreted proteins (numbers 1, 2, 3, 6, 7, 8 and 13 in Figure 8.8) were singled out on the basis that their relative abundance was shown to change following short-term local testicular heating. A summary of the molecular weights and isoelectric points of these proteins is given in Table 8 and the position of each is circled and labelled on each of the gels.

The secretion of proteins numbered 2, 6 and 13 correspond in molecular size and pI to three previously identified androgen-regulated (Sharpe *et al.*, 1992) and germ cell-dependent proteins (McKinnell and Sharpe, 1992). A further three proteins (numbered 1, 7 and 8) showed reductions in abundance following heat treatment 24 hours earlier. The identity of these three proteins is unknown although their secretion is known to be age-dependent i.e. their relative abundance was found to increase considerably with age (Chapter 4) show pronounced reductions in abundance following exposure to either m-DNB (Chapter 5), NB (Chapter 6) or MAA (Chapter 7). The secretion of protein 3 was found to have increased slightly following heat treatment 24 hours earlier. The identity of protein 3 is unknown but it may represent high pI forms of SGP-2.

8.4. Discussion

The experiments described in this chapter are the first to evaluate changes in the secretion of proteins by isolated ST following severe disruption of spermatogenesis, induced by short-term local testicular heating, and to equate

Protein No	M _r (kDa)	pI	Effect of local testicular heating	Stage-dependence of secretion	Possible Identity
1	63 - 70	5.6 - 5.8	Decrease	All stages	unknown
2	35 - 38.5	5.8 - 6.2	Decrease	VI-VIII	ARP-4 (Sharpe <i>et al.</i> , 1992)
3	28 - 30	5.8 - 6.9	Increase	All stages	Possible charge isomers of SGP-2
4	20 - 24	5.4 - 5.8	Decrease	All stages	unknown
6	20 - 30	6.9 - 7.4	Decrease	VI-VIII	ARP-3 (Sharpe <i>et al.</i> , 1992)
7	17 - 20	5.3 - 5.9	Decrease	All stages	unknown
8	16 - 20	5.9 - 6.4	Decrease	All stages	unknown
13	~9.5	6.9 - 7.4	Decrease	VI-VIII	ARP-1 (Sharpe <i>et al.</i> , 1992)

Table 8. List of seminiferous tubule secreted proteins which showed repeatable differences in relative abundance following short-term local testicular heating (43°C for 30 minutes). Protein numbers correspond to those shown in Figures 8.7 and 8.8.

Figure 8.7. Incorporation of ^{35}S -methionine into newly synthesized proteins secreted *in-vitro* by seminiferous tubules isolated at stages VI-VIII from control rats (top) or rats exposed to short-term local testicular heating (43°C for 30 minutes) 4 hours earlier. Proteins which change in relative abundance following local testicular heating 24 hours earlier (see Figure 8.8) are circled and numbered. The positions of the three major Sertoli cell secreted proteins, sulphated glycoprotein-1 (SGP-1), sulphated glycoprotein-2 (SGP-2) and cyclic protein-2 (CP-2), are indicated for reference.

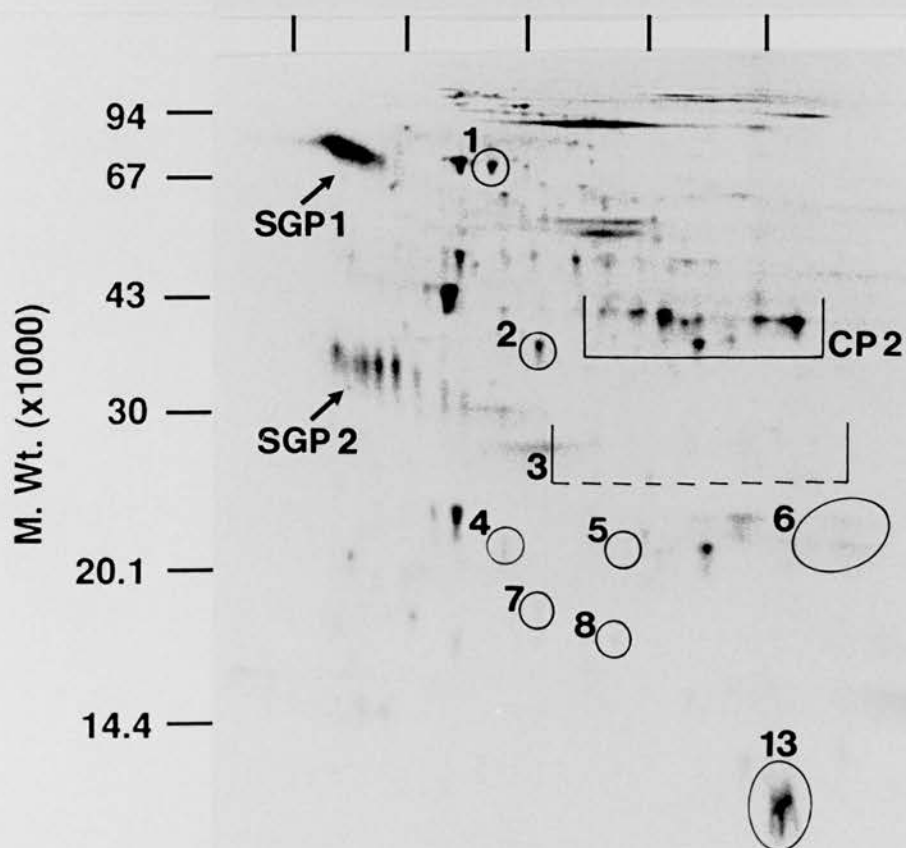
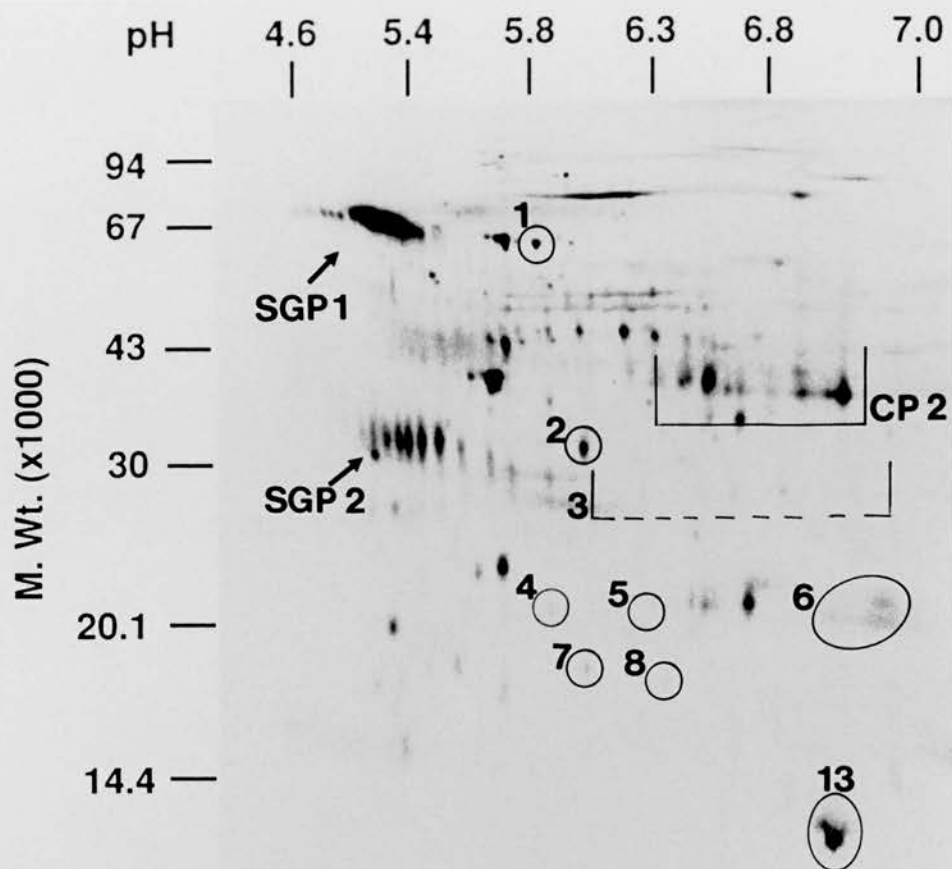
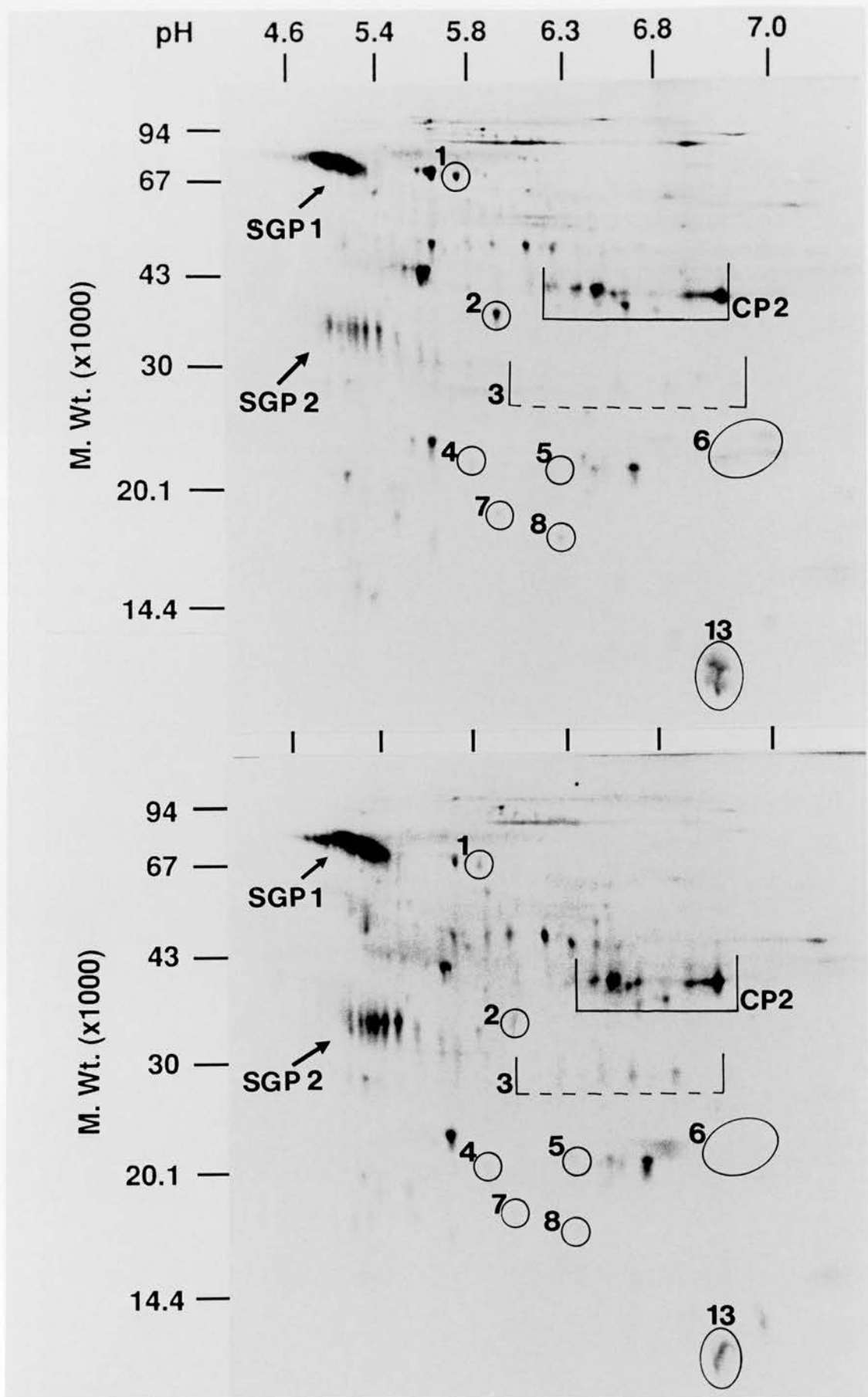


Figure 8.8. Incorporation of ^{35}S -methionine into newly synthesized proteins secreted *in-vitro* by seminiferous tubules isolated at stages VI-VIII from control rats (top) or rats exposed to short-term local testicular heating (43°C for 30 minutes) 24 hours earlier (bottom). Proteins which change in relative abundance following local testicular heating are circled and numbered. The positions of the three major Sertoli cell secreted proteins, sulphated glycoprotein-1 (SGP-1), sulphated glycoprotein-2 (SGP-2) and cyclic protein (CP-2), are indicated for reference.



these with histological changes.

Disruption of spermatogenesis, induced by local testicular heating, causes an initial lesion which is both stage- and cell-specific (Chowdhury and Steinberger, 1964, 1970; Collins and Lacy, 1969), with spermatocytes being the cell type most sensitive to heat. In the present study, exposure to short-term local testicular heating, for a duration known to cause subsequently severe disruption of spermatogenesis (Bartlett and Sharpe, 1987), resulted in stage-specific changes in the overall incorporation of ^{35}S -methionine into both secreted and intracellular proteins by seminiferous tubules isolated 4 and 24 hours after treatment. When compared with controls, the overall level of incorporation of ^{35}S -methionine into both secreted and intracellular proteins by ST at stages VI-VIII (the androgen-dependent stages; Sharpe *et al.*, 1992) was increased significantly ($p < 0.001$ and $p < 0.01$, respectively) when isolated 4 hours after treatment (Figures 8.4 and 8.5), whereas there was no significant change in incorporation of radiolabel into either secreted or intracellular proteins by ST at stages II-V or IX-XII. As the secretion of proteins by ST at stages VI-VII in the control situation is approximately double that of ST at other stages, these findings may indicate that exposure of the testis to heat simply exaggerates this process at 4 hours by increasing general metabolic activity. However, by 24 hours after local testicular heating, secretion of proteins by ST at stages VI-VIII was no longer increased, but was decreased when compared with controls, effectively abolishing the normal stage-dependent increase in protein secretion which occurs at stages VI-VIII. The latter change may perhaps explain the particular sensitivity of ST at these stages to heat and it is noteworthy that within 24 hours of treatment of rats with m-DNB, NB or MAA, at doses known to cause subsequent disruption of spermatogenesis, precisely the same changes are observed (see Chapters 5, 6 and 7) i.e. the normal stage-dependent increase in protein secretion at stages VI-VIII fails to occur, despite the maintenance of normal levels of testosterone (Bartlett and Sharpe, 1987; Allenby, 1990). ST isolated from rats 24 hours after treatment also showed a significant decrease in incorporation of ^{35}S -methionine into intracellular proteins at all stages (II-V, VI-VII and IX-XII) although this effect was least marked at stages VI-VIII, (Figure 8.4). These results therefore provide biochemical confirmation of the present and previous morphological studies showing that a single exposure of short-term local testicular heating results in stage-specific disruption of spermatogenesis as determined morphologically (Chowdhury and Steinberger, 1964, 1970; Collins and Lacy, 1969; Bartlett and Sharpe, 1987).

In this study the morphological effects of local heating on the rat testis were identified at the histological level within 24 hours of treatment. These initial effects were manifest as the loss/degeneration of pachytene spermatocytes at stages II-XIV of the spermatogenic cycle; whilst the nuclei of elongate spermatids at stages VI-VIII were found to be embedded deep within the Sertoli cell cytoplasm. Round spermatids in stages I-VII appeared normal in comparison to controls. The morphological effects described in this chapter confirmed in general the previously reported findings identified at a histological level in the rat by Chowdhury and Steinberger (1964, 1970), Collins and Lacy (1969), and Bartlett and Sharpe (1987). The reason for the initial susceptibility of pachytene spermatocytes to the adverse effects of local testicular heating is unknown. However, treatment of rats with chemicals such as m-dinitrobenzene (Blackburn *et al.*, 1988; Allenby, 1990 and see Chapter 5), nitrobenzene (Bond *et al.*, 1981; Allenby, 1990 and see Chapter 6), ethylene glycol monomethyl ether (EGME) methoxyacetic acid (Chapin *et al.*, 1984; Creasy *et al.*, 1985, 1986; Bartlett and Sharpe, 1988) and an imidazole compound (De Martino *et al.*, 1985) have all been shown to have rapid degenerative effects on pachytene spermatocytes. Neither is it known why round spermatids should be unaffected initially by the adverse effects of local testicular heating (Chowdhury and Steinberger, 1964), but it is perhaps significant that these germ cells have high levels of a testis specific heat shock protein - 70 gene - related transcript (hst 70 RNA) (Krawczyk *et al.*, 1988). Heat shock proteins are a group of intracellular proteins that are synthesized by cells in response to elevated temperatures and other stress factors (Craig, 1985; Pelham, 1986; Schlesinger, 1986). The function of these proteins is not fully understood, although recent studies suggest that they may be involved in the stabilization and assembly of nascent proteins in various cellular compartments, as well as in the recovery of cellular structures following stress factors (Pelham, 1986).

The mechanism by which heat affects spermatogenesis adversely is unclear, but several possible contributing factors have been identified. Firstly, the results of a study by Waites and Setchell (1964) suggest that local heating of the testis causes an increase in the rate of metabolism. However, this increase in oxygen and energy consumption is not accompanied by an increase in blood flow. Thus, the testis enters a state of self-induced hypoxia. The function of a cell depends on its ability to extract and use the chemical energy locked within the structure of organic molecules. The production of energy, in the form of adenosine triphosphate (ATP), involves a complex

series of reactions, which take place on the inner mitochondrial membrane, known as oxidative phosphorylation. The consumption of oxygen by mitochondrial oxidative phosphorylation accounts for most of the oxygen used by the body. Thus, if the availability of oxygen is reduced, as is the case following local testicular heating, ATP cannot be generated, the result of which is presumably the degeneration of certain 'sensitive' germ cells.

The second factor contributing to cell death, following testicular heating, may be a disturbance in carbohydrate metabolism, for it has been shown that addition of glucose during the incubation of testicular tissue can prevent the decrease in protein synthesis, which occurs at temperatures above 30°C (Davis, 1969). Sertoli cells play an important role in providing energy substrates for the developing germ cells in the form of lactate and pyruvate via the oxidation of glucose (Jutte *et al.*, 1981). Available evidence indicates that certain enzymes, involved in the oxidation of glucose, are affected adversely following increased temperatures. For instance, Ewing and Schanbacher (1970) found that a significant decrease in total hexokinase activity of the cryptorchid rat testis occurred after 48 hours, when there was morphological evidence of germ cell damage. In addition, total and specific activity of phosphofructokinase was found to decrease within 8 hours of cryptorchidism (Ewing and Schanbacher, 1970). Phosphofructokinase is the rate-limiting enzyme in the glycolytic pathway, and, in view of the dependence of the mammalian testis upon glucose, the results of this study suggest that those testicular cells containing reduced phosphofructokinase activity are unable to metabolize glucose, thereby reducing the availability of energy substrates. The question that remains is why this particular enzyme activity should be sensitive to the adverse effects of heat.

Despite a significant increase in overall incorporation of ³⁵S-methionine into secreted proteins by seminiferous tubules isolated at stages VI-VIII at 4 hours after local testicular heating, analysis by 2-D SDS PAGE revealed that no major changes had occurred in the secretion of specific proteins (Figure 8.7). However, by 24 hours after local testicular heating, the secretion of seven proteins (numbered 1, 2, 3, 6, 7, 8 and 13 in Figure 8.8) was found to have changed. In most cases (proteins numbered 1, 2, 6, 7, 8 and 13) their secretion was reduced. Three of these proteins (numbered 2, 6, and 13) correspond in molecular size and pI to three previously identified androgen-regulated (Sharpe *et al.*, 1992) and germ cell dependent (McKinnell and Sharpe, 1992) proteins. The identity of proteins numbered 1, 7 and 8 is unknown, although a recent study by McKinnell and

Sharpe (1992) has shown that the secretion of proteins numbered 1, 7 and 8 was decreased when either pachytene spermatocytes or round spermatids were missing from the seminiferous tubule. This might indicate that the secretion of these three proteins is regulated by more than one type of germ cell. Taken together, these results suggest that proteins 1, 7 and 8 might be secretory products of pachytene spermatocytes and/or round spermatids or that they are secretory products of Sertoli cells, the secretion of which is dependent on the presence of pachytene spermatocytes and/or round spermatids. Furthermore, the secretion of proteins numbered 1, 7 and 8 has been shown to be affected adversely following exposure to m-DNB, NB and MAA (see Chapters 5, 6 and 7 respectively). The secretion of one protein (number 3) was shown to increase slightly following exposure to heat 24 hours earlier. The identity of this protein is unknown, although it is possible that it represents isomers of SGP-2 with a high pI. The secretion of protein 5, which could be induced following exposure to either m-DNB or NB (see Chapter 5 and 6 respectively), was found not to be induced in this study, following short-term local testicular heating.

In conclusion, the experiments presented in this chapter have shown that short-term local testicular heating causes stage-specific changes in the incorporation of ^{35}S -methionine into both secreted and intracellular proteins within 4 hours of treatment. Analysis by 2-D SDS PAGE allowed the identification of seven proteins which were affected adversely following heat treatment, and these changes may underlie the disruption of spermatogenesis which follows this treatment. These proteins have been shown to be affected similarly following toxicant exposure, which suggests that they may have potential use as specific markers indicating disruption of spermatogenesis.

9. Effects of toxicants on ST-secreted proteins in man

Preceding chapters have described the adverse effects of three testicular toxicants, namely, meta-dinitrobenzene, nitrobenzene and methoxyacetic acid, on seminiferous tubule-secreted proteins in the rat. This chapter compares the effects of the same toxicants on seminiferous tubule-secreted proteins in man in order to establish whether effects can be seen comparable to those observed in the rat (Chapters 5, 6 and 7).

9.1. Introduction

There is increasing evidence from studies in animals that many environmental chemicals in everyday use can potentially affect male reproductive function (Lucier *et al.*, 1977; Lamb and Foster, 1988), but there is very little evidence of such effects in man (Henderson *et al.*, 1986). To date, the rat is the species used most widely in studies of general pharmacology, endocrinology and male reproductive physiology, as well as in routine toxicity studies where the adverse effects of new chemicals and drugs on reproductive function are evaluated. The toxicity of chemicals is, however, known to vary considerably between different animal species (Torkelson *et al.*, 1961) and, accordingly, an extrapolation from rat to man cannot always be assumed safely. Thus, the aim of the experiments described in this chapter was to compare the effects of three testicular toxicants, which have known adverse effects on spermatogenesis in the rat, on isolated rat and human seminiferous tubules. The compounds tested were m-DNB (Foster *et al.*, 1986; Blackburn *et al.*, 1988; Allenby, 1990 and see Chapter 5) and NB (Bond *et al.*, 1981; Levin *et al.*, 1988; Allenby *et al.*, 1990; and see Chapter 6), both of which have been established to be testicular toxicants and which act directly on the Sertoli cell, and MAA which is a well-established testicular toxicant thought to act directly on pachytene spermatocytes, (Creasy *et al.*, 1985; Bartlett *et al.*, 1988; and see Chapter 7).

9.2 Experimental procedures

9.2.1. Preparation of human ST

Human testicular tissue was placed on ice immediately after excision and transported from the Western General Hospital, Edinburgh, to the MRC Reproductive Biology Unit, Edinburgh as quickly as possible (usually within 30-60 minutes of removal). The testis was weighed and cut into pieces

of approximately 10mm³, following which 8-10mg of human tissue was placed into a 25ml universal container with 7mls incubation medium; consisting of M199H (Flow Labs) supplemented with 0.75mg/ml collagenase (Worthington), 0.1% (v/v) trypsin inhibitor and 2.5mg/ml BSA (both from Sigma). The testicular tissue was incubated in a shaking water bath at 70 cycles/minute for 45-60 minutes at 37°C. The universals were arranged so that the shaking movement was longitudinal. The contents of each universal were diluted subsequently with dispersion medium, which consisted of M199H (Flow Labs) containing 0.5mg/ml BSA (Sigma), and swirled manually forty times. Tissue and tubule fragments were allowed to settle and the resultant supernatant removed carefully and discarded. The tissue remaining was rediluted with dispersion medium and swirled manually thirty times, following which the resulting seminiferous tubules were isolated as described in 9.2.2. All of the above procedures were performed kindly by Dr Safia Qureshi.

9.2.2. Procedure for isolation of human ST

Following digestion, small pieces of human tissue were placed into a small plastic Petri dish containing ice cold Dulbecco's phosphate buffered saline (PBS: Flow Labs) on a cooled (4°) transparent perspex stage illuminated from below. Lengths of seminiferous tubules were then isolated by teasing apart gently the central portion of tissue using fine watchmakers' forceps. Unlike seminiferous tubules isolated from rats, human seminiferous tubules are much finer and 'stickier', which makes it impossible to measure accurately the lengths of tubules being isolated. As it is impossible to distinguish by illumination the various stages of the spermatogenic cycle, using normal criteria, which rely on changes in the density and position of the heads of elongate spermatids (Parvinen, 1982) a pool of unstaged human ST was isolated. ST were then cultured for 24 hours in the presence or absence of m-DNB, NB or MAA (all at 10⁻⁴M) according to the methods described in Chapter 3.

9.2.3. Procedure for isolation of ST from control rats

Seminiferous tubules were isolated from untreated control rats and cultured for 24 hours in the presence or absence of m-DNB, NB or MAA (all at 10⁻⁴M) according to the methods described in Chapter 3.

9.2.4. Treatment of cultures

m-DNB (Aldrich Chemical Co), NB (Sigma) or MAA (Aldrich Chemical Co) were added to cultures at a concentration of 10^{-4} M in dimethyl sulfoxide (DMSO : BDH) at a final concentration of 0.3% (v/v), while controls received an equal volume of this vehicle. The concentrations of m-DNB, NB and MAA were chosen as being approximately equivalent to the peak levels, achieved *in-vivo* following administration of 50mg/kg m-DNB, 300mg/kg NB or 500mg/kg MAA (see Allenby *et al.*, 1991b for discussion and references), which are known to cause severe disruption of spermatogenesis. Treatments caused no appreciable alteration of the culture medium pH.

9.2.5. Measurement of newly synthesized proteins

Incorporation of 35 S-methionine into secreted proteins was determined by precipitation of aliquots of the culture medium with 10% (v/v) trichloroacetic acid, according to the methods described in Chapter 3.

9.2.6. Two-dimensional SDS PAGE

Newly synthesized 35 S-methionine labelled proteins secreted by isolated human and rat ST were evaluated by 2-D SDS PAGE according to the methods described in Chapter 3.

9.3. Results

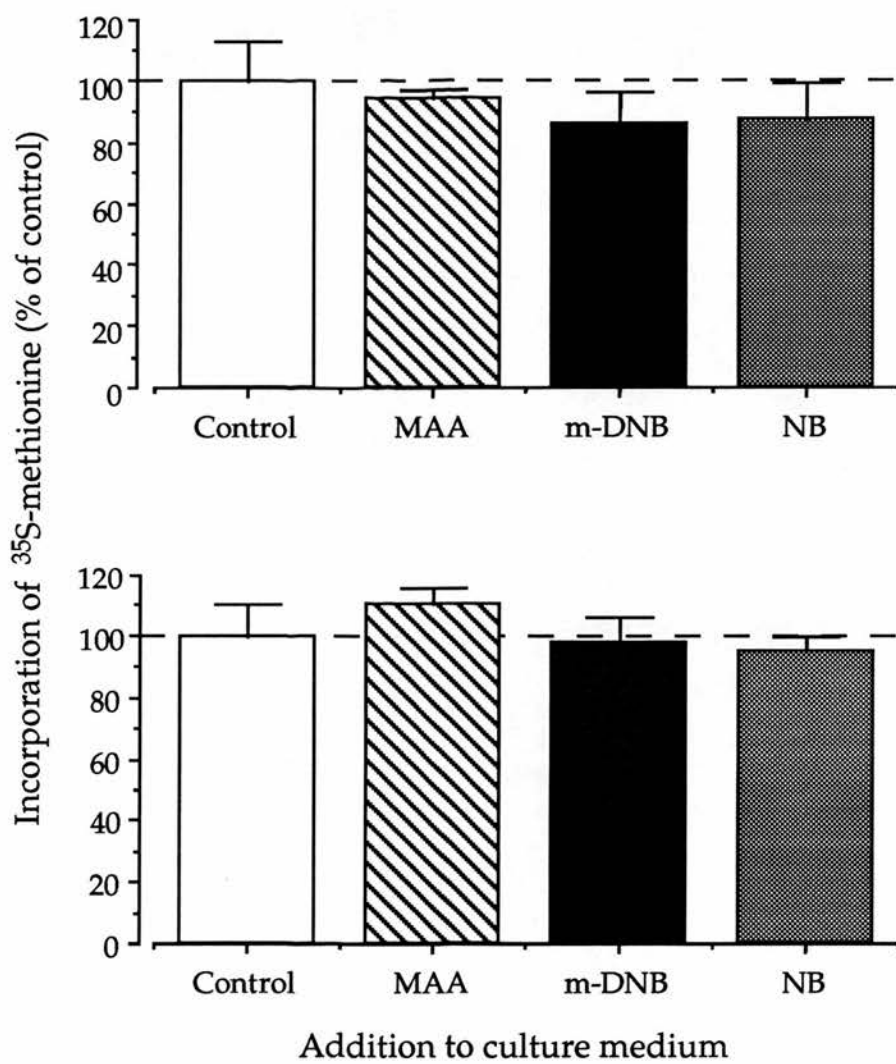
9.3.1. Effect of addition of toxicants to human and rat ST

The effect of the addition of m-DNB, NB or MAA to ST isolated from either rats or humans is shown in Figure 9.1. The addition of m-DNB, NB or MAA *in-vitro* to ST isolated from rats caused a slight, but statistically insignificant, decrease in incorporation of 35 S-methionine into secreted proteins (Figure 9.1). The same additions to isolated human ST had no effect on incorporation (Figure 9.1).

9.3.2. Two-dimensional SDS PAGE

Analysis, by 2-D SDS PAGE, of newly synthesized proteins secreted by rat and human ST is shown in Figure 9.2. Substantial differences were observed between the pattern of proteins secreted by ST isolated from adult rats when compared with the pattern of proteins secreted by human ST (Figure 9.2). It

Figure 9.1. Incorporation of ^{35}S -methionine over 24 hours into newly synthesized proteins secreted *in-vitro* by unstaged seminiferous tubules isolated from untreated rats (top) or humans (bottom) and cultured in the absence (control) or presence of either 10^{-4}M m-DNB, NB or MAA. Incorporation has been expressed as a percentage of the mean control value (dashed line) obtained during the same experiment. Values are expressed as the \pm SD of $n=3$.



is emphasized that all of these differences were confirmed in separate gels using medium from 3 separate experiments. In general, remarkably little variation was found between experiments. Many of the differences related to proteins that were secretory products of rat, but not of human, ST. Five proteins (numbered 1, 2, 4, 7 and 8), which were detectable secretory products of adult rat ST, were not secreted by human ST. Three major Sertoli cell proteins, namely SGP-1, SGP-2 and CP-2 (Griswold, 1988), which are prominent secretory products of rat ST, were found on the basis of their molecular weight and pI to be secreted by human ST. A further five proteins (numbered 6, 14, 15, 16 and 17) were found to be secreted by both rat and human ST. Protein number 6 corresponds to a recently identified androgen-dependent protein (Sharpe *et al.*, 1992). Protein 5, which had a molecular weight of approximately 26 kilodaltons, was not secreted by normal adult rat ST but its secretion could be induced by culture of the ST for 24 hours with m-DNB (Figure 9.3). The secretion of protein 3, which exists as a prominent band of charge isomers of approximately 28-30 kilodaltons, may represent high pI forms of SGP-2 in humans. Exposure of ST from adult rats to m-DNB caused the disappearance of, or a substantial decrease in, the secretion of all of the proteins described except for numbers 2, 3 and 5, but had relatively little effect on proteins secreted by isolated human ST, with the exception of protein 5 which was increased slightly (Figure 9.3).

9.4. Discussion

The experiments described in this chapter are the first to evaluate protein secretion by isolated human ST, and how this is influenced following exposure to known testicular toxicants. In addition, a comparison was made of the profile of protein secretion by isolated rat and by human ST *in-vitro*.

To date our knowledge of Sertoli cell function in humans is rather limited, and only a few reports have been published (Lipshultz *et al.*, 1982; Holmes *et al.*, 1984; Santiemma *et al.*, 1985). In general, most of the work on the function of human Sertoli cells has concentrated on studying the existence of factors that modulate Leydig cell steroidogenesis *in-vitro* (Verhoeven and Cailleau, 1987; Papadopoulos *et al.*, 1991; Lejeune *et al.*, 1993). The majority of these *in-vitro* studies have used mainly primary cultures of Sertoli cells. As has been discussed previously, this may not be the most satisfactory approach, especially as it now appears that the presence of germ cells (and other cell types) found normally in the testis may serve to modify Sertoli cell

Figure 9.2. Comparison using 2-dimensional SDS PAGE of proteins secreted by unstaged ST isolated from rats aged 70 (top) days or humans (bottom). Proteins which have comparable M_r s have been circled and numbered. The positions of the three major Sertoli cell secreted proteins, sulphated glycoprotein-1 (SGP-1), sulphated glycoprotein-2 (SGP-2) and cyclic protein-2 (CP-2), are indicated for reference.

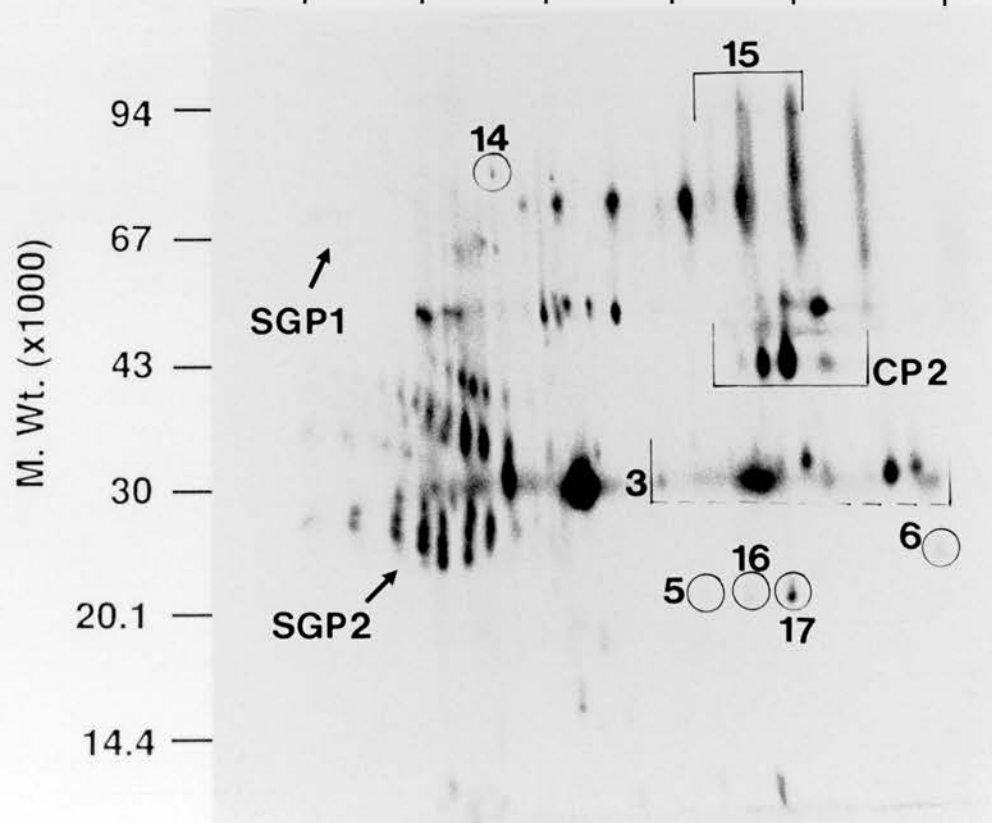
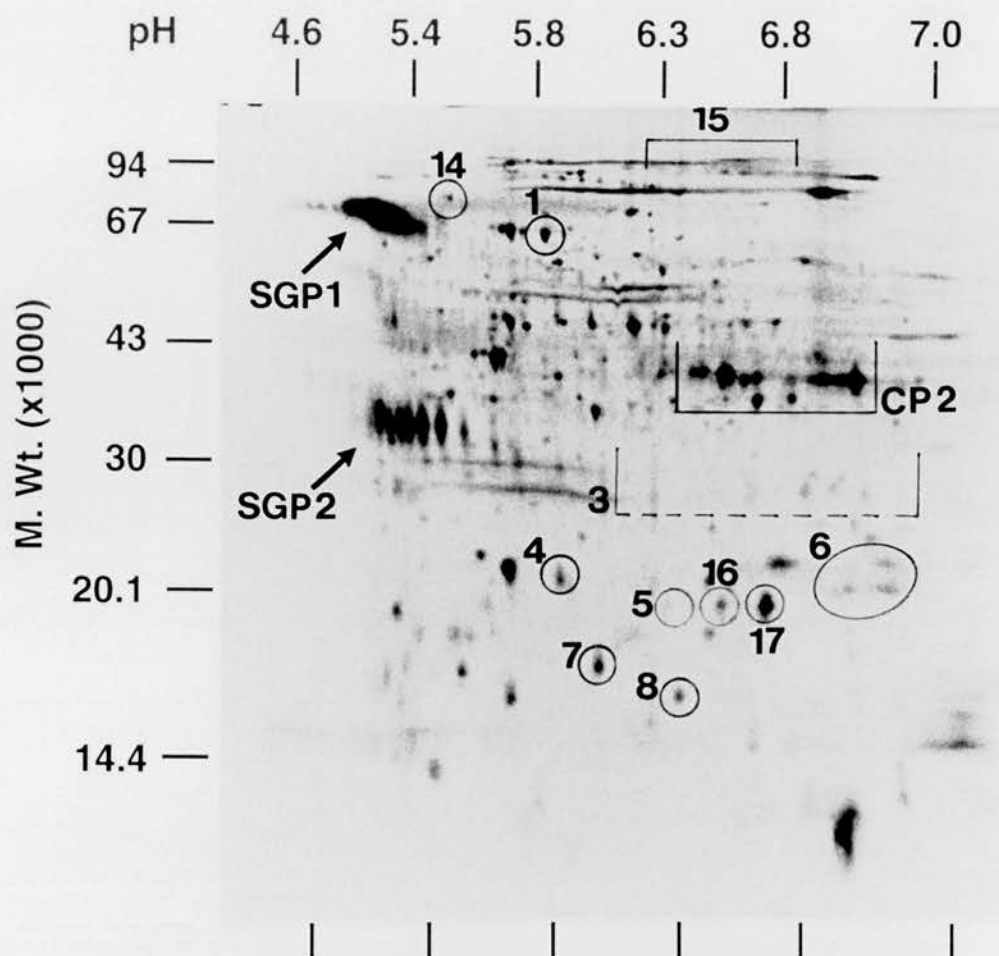
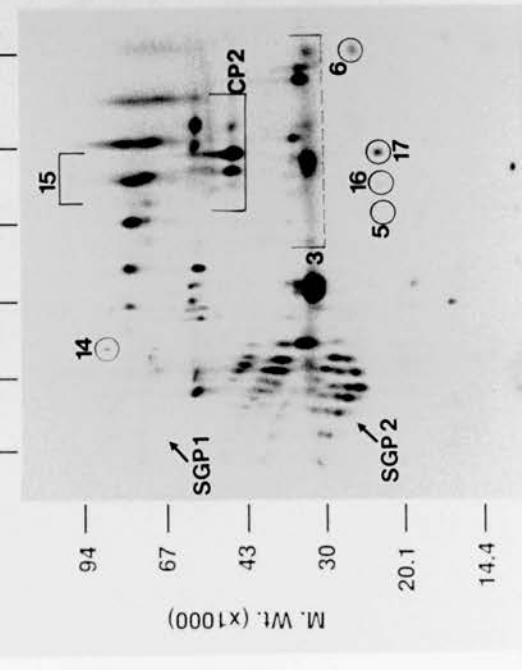
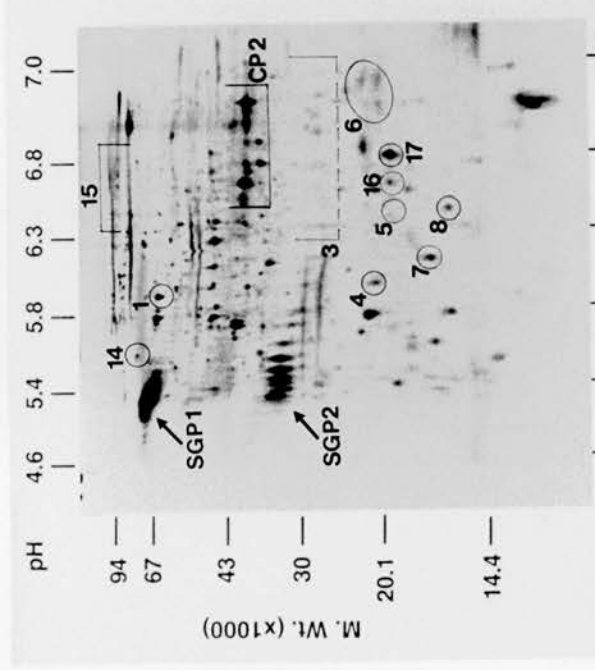
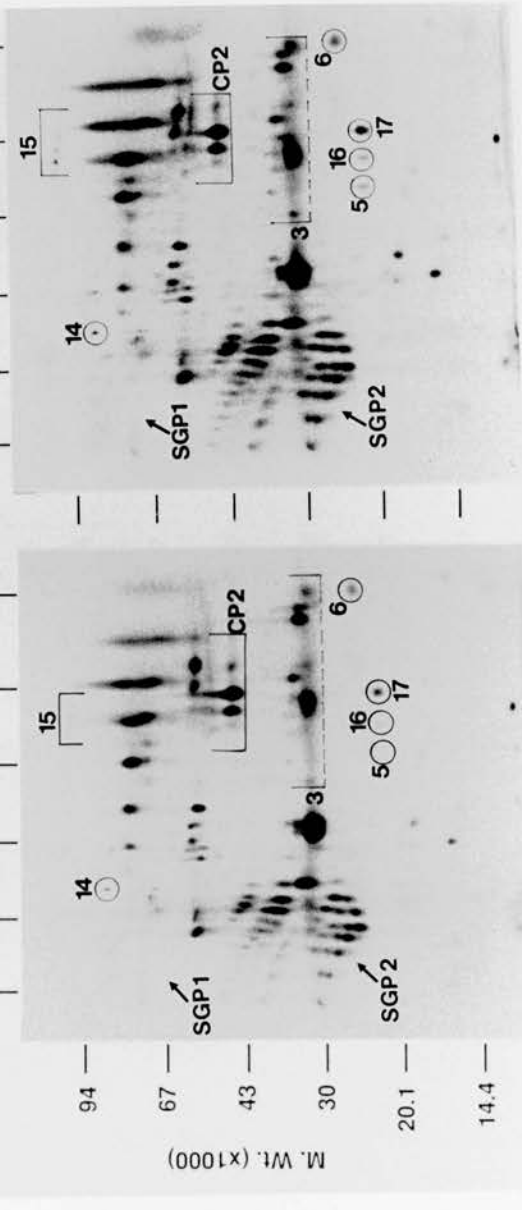
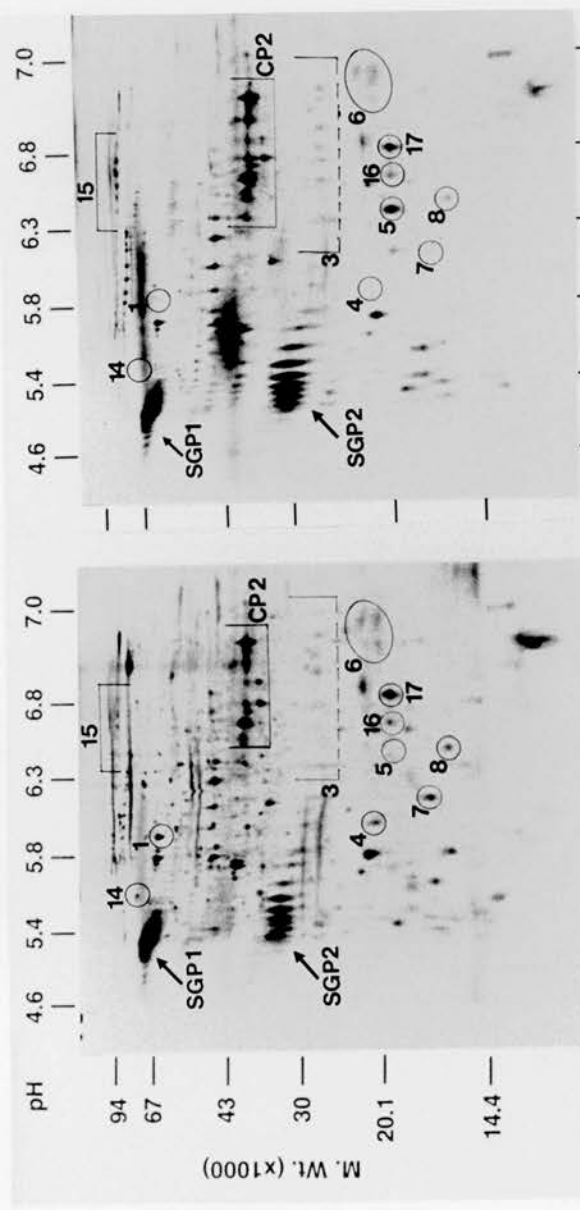


Figure 9.3. Comparison using 2-dimensional SDS PAGE of the effect of m-DNB (10^{-4}M) on proteins secreted by unstaged ST isolated from rats aged 70 (top) days or humans (bottom) and cultured in the absence (left) or presence (right). Proteins which have comparable M_r s and which changed in relative abundance following m-DNB exposure are circled and numbered. The positions of the three major Sertoli cell secreted proteins, sulphated glycoprotein-1 (SGP-1), sulphated glycoprotein-2 (SGP-2) and cyclic protein-2 (CP-2), are indicated for reference.



function (Sharpe, 1993). In this study, human ST were used because they probably maintain, as far as is possible, the cell-cell interactions which are found normally *in-vivo*. The present study has also evaluated the susceptibility of isolated human ST to the effects of three well-characterized testicular toxicants, m-DNB (Foster *et al.*, 1986; Blackburn *et al.*, 1988; Hess *et al.*, 1988; Allenby, 1990) and NB (Bond *et al.*, 1981; Levin *et al.*, 1988; Allenby *et al.*, 1990), both of which have been shown to exert their adverse effects on spermatogenesis via perturbation of the Sertoli cells, and MAA, a germ cell specific toxicant which causes selective and stage-specific depletion of pachytene spermatocytes (Foster *et al.*, 1983; Creasy *et al.*, 1985; Bartlett *et al.*, 1988).

The addition of m-DNB, NB or MAA to ST isolated from adult rats caused a slight, but statistically insignificant, decrease in incorporation of ^{35}S -methionine into overall secreted proteins (Figure 9.1), whereas the same additions to isolated human ST had no effect on incorporation of radiolabel into overall secreted proteins (Figure 9.1). Exposure to m-DNB, NB and MAA either *in-vitro* or *in-vivo* has been shown in previous chapters to cause stage-dependent changes in overall protein secretion (see Chapters 5, 6 and 7 respectively). In the present study unstaged ST were used as it is impossible to stage human ST using the normal criteria, which rely on changes in the density and position of the heads of elongate spermatids (Parvinen, 1982). As a consequence of using unstaged ST, any stage-dependent decreases in overall protein secretion may be masked.

Analysis of ST-secreted proteins by 2-D SDS PAGE revealed substantial differences in the proteins secreted by isolated rat and human ST and in their relative abundance. Three major Sertoli cell proteins, namely SGP-1, SGP-2 and CP-2 (Griswold, 1988) which are prominent secretory products of rat ST were found to be secreted, to a lesser or greater degree, by human ST on the basis of their molecular weight and pI. Five proteins (numbered 1, 2, 4, 7 and 8) which were detectable secretory products of adult rat ST, were not-detectable as secretory products of human ST at least on the basis of M_r and pI. A further five proteins (numbered 6, 14, 15, 16, 17) were found to be secreted by both rat and human ST. The secretion of proteins numbered 2 and 6 is known to be acutely androgen-dependent (Sharpe *et al.*, 1992) and germ cell regulated (McKinnell and Sharpe, 1992) in the adult rat; these proteins were not particularly prominent in the present study because unstaged ST were used. Protein 5 was secreted by isolated human ST but was not secreted by control adult rat ST, although it has been shown to be secreted

by ST isolated from control immature animals (see Chapter 4). The secretion of protein 3, shown in this study to be a prominent feature of isolated human ST, has been shown to be prominent in SC+GC co-cultures from immature rats, still prominent but less so in ST from immature rats and absent or of very low abundance in the adult (see Chapter 4). The identity of protein 3 is unknown though it is possible that it represents isomers of SGP-2.

Exposure of adult rat ST *in-vitro* to m-DNB induced a marked decrease in the secretion of five specific proteins (numbered 1, 4, 6, 7 and 9), most of which were undetectable, or were only minor, components of isolated human ST. In contrast, protein 5 increased in abundance following culture of human and rat ST with m-DNB, though this protein was not detectable in adult rat controls. The secretion of protein 3 was also shown to increase following exposure of rat ST to m-DNB. The secretion of proteins 3 and 5 is thought to be germ cell dependent i.e. their secretion increases when a particular germ cell type(s) (are) is absent from the ST (see Chapter 4). Furthermore, removal of two generations of germ cells from the ST of adult rats leads to a substantial increase in secretion of protein 3 (McKinnell and Sharpe, unpublished data). Human ST even when isolated from young men, are known to possess fewer germ cells than rat ST (Johnson, 1986). In this study the ST used were isolated from aged men. These ST, although they have been shown to have complete spermatogenesis, have significantly reduced numbers of germ cells and show signs of Sertoli cell dysfunction. It may be suggested therefore, that the secretion of proteins 3 and 5, which were found in this study to be a prominent feature of human ST, may be due in part to the decreased number of germ cells and/or Sertoli cell dysfunction.

In conclusion, the experiments presented in this chapter have shown that the pattern of protein secretion of rat and human ST, and their response to testicular toxicants, is somewhat different. On the basis of these findings it is therefore possible that the rat is not a particularly good model for investigating both testicular toxicity and Sertoli cell function in man. It must be remembered, however, firstly, that this comparison is based on protein identification based on M_r and pI and, second, that the ST used in this study, were from aged men, and that ST isolated from young men may give different results.

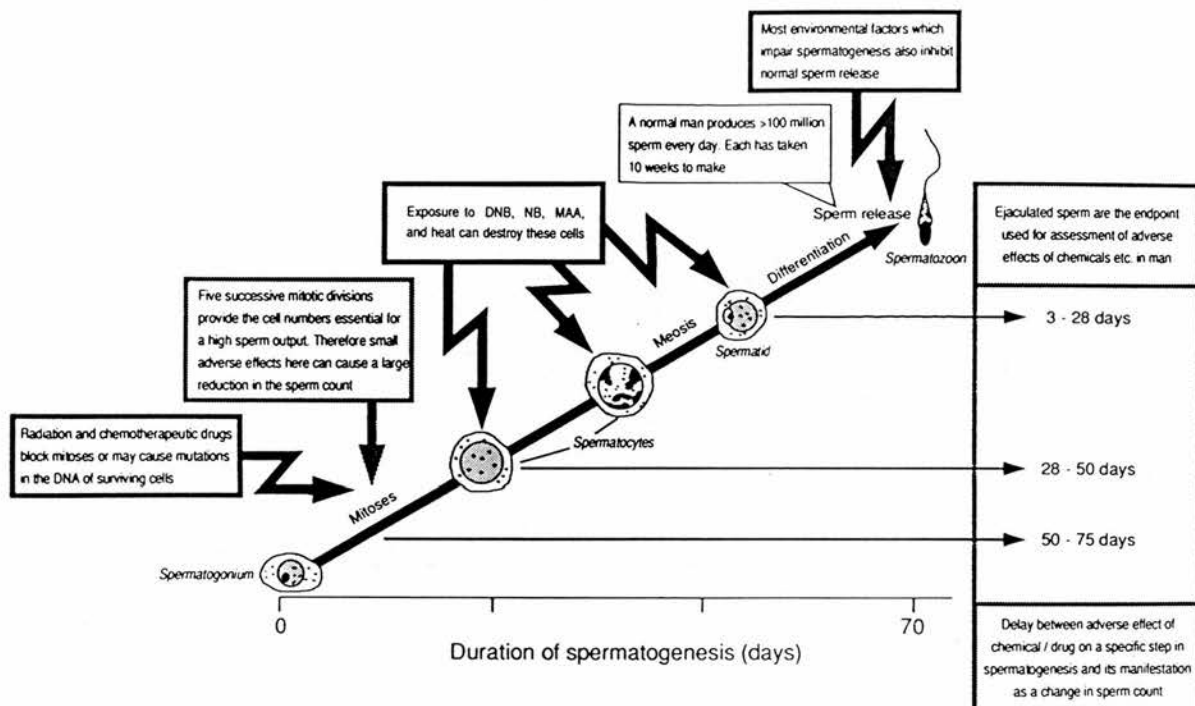
10. General Discussion

There is growing concern about the adverse effects of environmental chemicals on male reproductive function, since a recent study by Carlsen *et al.* 1992 has shown that mean sperm counts in normal western men have fallen by 40-50% over the last 50 years. It remains unknown to what extent environmental exposure has contributed to this overall decrease because, at present, we lack sensitive methods with which to identify adverse effects of chemicals on spermatogenesis.

From studies in animals, and in the experiments described in this thesis, many chemicals or environmental factors (e.g. heat) which affect spermatogenesis adversely do so in a very selective manner (Chowdhury and Steinberger, 1970; Creasy and Foster, 1984; Meistrich, 1986; Bartlett and Sharpe, 1987; Blackburn *et al.*, 1988; Allenby, 1990). Furthermore, most of these adverse changes affect earlier rather than later germ cell types and, accordingly, the earlier in the process of spermatogenesis that a chemical acts, the longer will be the delay before it is manifest as a decrease in sperm count (Figure 10.1). This highlights the need for more sensitive methods with which to detect early adverse effects of chemicals on spermatogenesis. Thus, the principal aim of this thesis was to identify potential markers of early toxicant-induced damage to spermatogenesis, by evaluating changes in the secretion of specific proteins from isolated ST following toxicant exposure. The identity of such proteins highlights not only the possible biochemical causes of the toxic effects of chemicals on the testes but also enables identification of some of the important biochemical steps in spermatogenesis. In the long term it is hoped that such protein markers could be used in routine toxicological and clinical studies to provide a better and more sensitive indicator of hazard to male reproductive ability, and that they will have a major application in basic and clinical studies of fertility regulation in the male. ST-secreted proteins were targetted specifically because it is widely accepted that Sertoli cells orchestrate and control the process of spermatogenesis via the secretion of specific proteins and because it is believed that the secretion of these proteins represents the most important of the possible routes by which Sertoli cells communicate, and thus interact, with germ cells (Sharpe, 1993).

The culture of isolated Sertoli cells *in-vitro* has become an increasingly popular laboratory technique for investigating both testicular toxicity and Sertoli cell function. From the perspective of the reproductive toxicologist the

Figure 10.1. Illustration of the time-scale of spermatogenesis in man and the steps in the process which are susceptible to the adverse effects of chemicals and other environmental factors such as heat. The temporal delay between induction of such adverse effects and their consequent manifestation as a change in sperm count in the ejaculate is shown in the box to the right. (Courtesy of R. Sharpe).



use of such *in-vitro* culture systems has undoubtedly aided the investigation of the mechanism and site of action of a number of testicular toxicants, including m-DNB, NB and MAA. There are, however, several disadvantages to primary testicular cell culture, when used in the context both of examining Sertoli cell function and of identifying potential testicular toxicants. Firstly, the vast majority of studies have used Sertoli cells from immature animals and, as these cells are known to change many of their functions as they mature during puberty (Jégou, 1991), the relevance of this data to the normal adult is often not assessed (Russell and Steinberger, 1989). Secondly, most of these studies have used Sertoli cells which are co-cultured with a particular class of germ cells. In view of the complexity of cell-cell interactions in the testis it must be remembered that, although a particular type of germ cell may modify particular functions of immature cells *in-vitro* (Galdieri *et al.*, 1984; Le Magueresse and, Jégou, 1986; Castellón *et al.*, 1989), the presence of all of the other cell types that occur in the normal adult testis, may modify this interaction even further.

With these disadvantages in mind, the experiments described in Chapter 4 were designed to assess the effect of age on protein secretion by isolated ST and to assess whether the protein response to the addition of known testicular toxicants or hormones is comparable at different ages. Due to the disadvantages in the use of isolated Sertoli cells, which have been discussed previously, ST were used because they maintain, as far as is possible, the cell-cell interactions which are found normally *in-vivo* and because, unlike Sertoli cells, ST can be isolated with comparable ease from both immature and mature rats. The data presented in this chapter showed quite clearly that both the pattern and the total level of protein secretion differed markedly between immature and adult rats, as did their response to toxicants and FSH. It is presumed that these differences are mediated by the presence of more mature germ cells within the ST and changing Sertoli cell function as a result of increasing age. These results add to the growing evidence suggesting that germ cells are capable of modulating the secretory functions of the Sertoli cell, according to their own specific requirements. Moreover, these findings have widespread implications for studies of the regulation and modulation of spermatogenesis and suggest that more effort should be directed towards studies of the adult than the immature rat. Accordingly, all subsequent experiments described in this thesis were performed on adult rats.

Although we lack detailed understanding of the control of spermatogenesis, it is generally accepted that the various steps in germ cell multiplication, meiosis and differentiation are driven by proteins secreted by the Sertoli cells and, further, that the secretory function of the Sertoli cell varies cyclically according to the stage of the spermatogenic cycle (Parvinen, 1982, 1993). It is presumed that some of these functions are either altered or are more sensitive to the adverse effects of particular chemicals or treatments. Therefore, the early effects of two known Sertoli cell toxicants, meta-dinitrobenzene (m-DNB) and nitrobenzene (NB), on the secretion of both overall and specific proteins by ST isolated from adult rats at different stages of the spermatogenic cycle, were assessed. The aim of using these established and relatively well-understood toxicants was to enable markers to be identified which can be related to toxic damage to a particular cell type. Within 24 hours of a single oral administration of either m-DNB or NB, stage-specific changes in overall protein secretion were observed. Similar results were also obtained with ST that had been isolated from untreated control rats and cultured in the presence of m-DNB or NB (10^{-4} M) for 24 or 72 hours. Analysis of ST-secreted proteins by 2-D SDS PAGE identified 10 marker proteins which showed major reproducible changes in secretion following exposure to either m-DNB or NB. Of particular interest was the finding that, following exposure to either m-DNB or NB, the secretion of a 26kDa protein (number 5) could be induced. Morphological data have shown that, by 24 hours after administration of either m-DNB or NB to adult rats, there is massive loss or degeneration of pachytene spermatocytes at stages VI-XII, whereas stages II-V of the spermatogenic cycle are not affected until 72 hours. The presently observed protein changes therefore either precede (stages II-V) or accompany (stages VI-XII) germ cell degeneration, which suggests that they may have potential use as markers of early toxicant-induced damage to spermatogenesis, and/or that the changes in these proteins may mediate some of the adverse testicular effects of m-DNB and NB.

The effects of methoxyacetic acid (MAA) on seminiferous tubule protein secretion were also assessed. MAA is a well-established germ cell specific toxicant, which causes the selective and stage-specific destruction of pachytene spermatocytes. The normal progress of the remaining germ cells through spermatogenesis, coupled with the evidence which suggests that MAA acts directly on the pachytene spermatocytes (rather than via perturbation of the Sertoli cell e.g. m-DNB and NB), means that any changes

observed at various time points after MAA-treatment are a specific consequence of depletion of the germ cell type in question. Following a single oral administration of MAA, stage-specific changes in overall protein secretion, similar to those observed following exposure to m-DNB and NB, were observed following exposure to MAA. Analysis of ST-secreted proteins by 2-D SDS PAGE identified seven proteins which were found to change following exposure to MAA; all of these proteins had been affected by exposure to m-DNB or NB. Of particular interest was the finding that the secretion of protein 5, which could be induced following exposure to m-DNB or NB, could not be induced following exposure to MAA. These results therefore suggest that protein 5 might be a specific marker of Sertoli cell dysfunction. The secretion of this protein has been shown, however, to be secreted normally by Sertoli cell+germ cell co-cultures and ST when isolated from immature rats.

In order to assess whether the changes seen in protein secretion, following exposure to m-DNB, NB or MAA were specific markers of toxicant-induced damage to spermatogenesis, the effects of severe disruption of spermatogenesis, induced by short-term local testicular heating, were assessed so as to establish whether protein changes comparable to these seen following toxicant exposure could be induced. Within 4 hours of treatment, stage-specific changes in the incorporation of ^{35}S -methionine into both secreted and intracellular proteins were observed, and these changes may underlie the disruption of spermatogenesis which follow this treatment. Analysis by 2-D SDS PAGE identified seven proteins which were affected adversely following heat treatment, all of which had been shown previously to be important predictors of cell-and stage-specific damage to spermatogenesis (see Chapters 5, 6 and 7). This suggests that these proteins may have potential use as specific markers indicating disruption of spermatogenesis. The secretion of protein 5, which could be induced following exposure to either m-DNB or NB, was found not to be induced by short-term exposure to heat. The findings of this study suggest further that the 'appearance' of protein 5 may be a specific marker of Sertoli cell dysfunction. In order to confirm this suggestion, further studies using other known Sertoli cell toxicants (for example, mono-(2-ethylhexyl) phthalate (MEHP); (Foster *et al.*, 1982), are required.

Having identified significant reproducible changes in protein secretion in the rat, following exposure to m-DNB, NB or MAA, the effects of these

toxicants on isolated ST were examined in order to establish whether comparable protein changes could be induced in man (Chapter 9). It was found generally that toxicant exposure caused no change in the incorporation of ^{35}S -methionine into overall secreted proteins. Several differences were observed between the pattern of proteins secreted by ST isolated from adult rats and the pattern of protein secretion by human ST. Furthermore, it was found that toxicant exposure caused very little change in the secretion of specific proteins by human ST, with the notable exception of protein 5, which was induced similarly in rat and man following exposure to m-DNB. On the basis of these results it would seem that man is less sensitive than the rat to the adverse effects of the testicular toxicants used in these studies. It must be remembered that the ST used in these studies were from aged men and, although these ST have been shown to have complete spermatogenesis, they have significantly reduced numbers of germ cells. It is possible therefore that ST isolated from younger men may have given different results.

From the studies presented in this thesis, it is not possible to state whether the 'marker proteins' emanate from the Sertoli, peritubular or germ cells. In order to identify the possible cellular source of these proteins, future experiments could use m-DNB or NB to induce Sertoli cell dysfunction *in-vivo* in order to investigate *in-vitro* the secretory function of isolated Sertoli cells. In addition Sertoli cells could be isolated from untreated control rats and then cultured in the presence or absence of the same toxicants. Furthermore, the effects of m-DNB, NB or MAA on isolated germ cell preparations enriched in either pachytene spermatocytes or round spermatids could be studied to identify whether any of the marker proteins originate from these cells. It must be recognized, however, that the use of isolated cells has two main limitations; a) their secretory function might alter after isolation from their normal environment, and b) isolated cells might include cells from ST at stages which are not affected initially by toxicant exposure.

The results of the studies presented in this thesis have established and confirmed the validity of the approach required to identify potential markers of disruption to spermatogenesis, and have identified several likely candidates. In order to establish the usefulness of these protein markers, they will first have to be purified and isolated. Following their isolation the usefulness of the protein(s) as a marker *in-vivo* will require to be assessed, by comparing its level in testicular IF, rete testis fluid and testicular, epididymal, spermatic and peripheral venous blood (Maddocks and Sharpe, 1989). This

will enable demonstration of whether the protein marker is absorbed from the reproductive tract (and, if so, from where) and whether it then appears in blood, as would be predicted (Sharpe, 1989).

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